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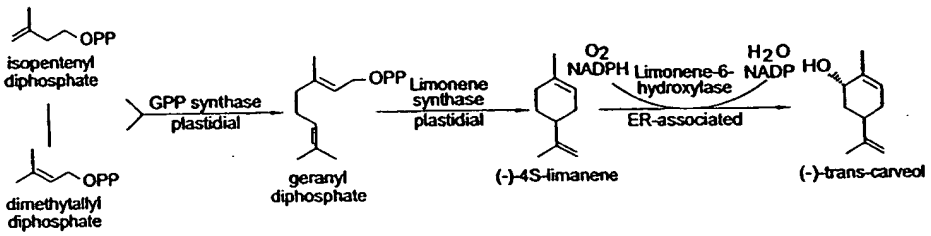
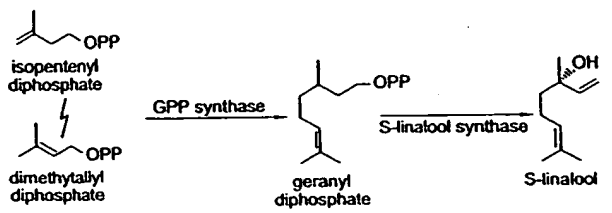
(54) Title: LIMONENE AND OTHER METABOLITES OF GERANYL PYROPHOSPHATE FOR INSECT CONTROL





(57) Abstract: Methods for manipulating metabolic pathway in plants, particularly those pathways involved in the biosynthesis of monoterpenes are provided. Methods are directed at transforming plants with one or more nucleotide sequences encoding the enzyme GPP synthase, and the monoterpene synthases limonene-, carveol and S-linolool synthase. Methods for creating or enhancing resistance to insects in plants by transforming plants with GPP- and/or monoterpene synthases, to generate plants producing monoterpenes in amount effective for resistance to insects are also provided.

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LIMONENE AND OTHER METABOLITES OF GERANYL PYROPHOSPHATE FOR INSECT CONTROL

FIELD OF THE INVENTION

This invention relates to methods for genetic manipulation of metabolic pathways in plants, particularly to transforming plants with genes involved in monoterpene biosynthesis and resistance to insects.

BACKGROUND OF THE INVENTION

Numerous insects are serious pests of common agricultural crops. One method of controlling insects has been to apply insecticidal organic, semiorganic or organometallic chemicals to crops. This method has numerous, art-recognized environmental and public health problems. A more recent method of control of insect pests has been the use of biological control organisms which are typically natural predators of the troublesome insects. These include other insects such as trachonid wasps, fungi such as *Beauveria Bassiana*, and bacteria such as Bacillus thuringiensis cv., commonly referred to as "Bt". However, it is difficult to apply biological control organisms to large areas, and even more difficult to cause those living organisms to remain in the treated area for an extended period. Still more recently, techniques in recombinant DNA have provided the opportunity to insert into plant cells cloned genes which express insecticidal toxins derived from biological control organisms such as Bt. This technology has given rise to concerns about eventual insect resistance to well-known, naturally occurring insect toxins, particularly in the face of heavy selection pressure, which may occur in some areas. Thus, a continuing need exists to identify naturally occurring insecticidal toxins which can be formed by plant cells directly by expression of structural genes not normally present in the plant.

Southern Corn Rootworm (*Diabrotica undecimpunctata howardi* Barber) is a particularly difficult pest to control or eradicate. It attacks the plant below the soil line, where insecticides are difficult or impossible to apply effectively. In addition, it is resistant to a number of otherwise effective chemical and biological control agents, including Bt toxins and some lectins.

The monoterpene, limonene, 1-methyl-4 (1-methylethenyl) cyclohexene; p-mentha-1,8-diene (Entry No. 5371, Merck Index 11th Ed.), occurs naturally in various ethereal oils, particularly oils of lemon, orange, caraway, dill and bergamot. It is a valuable industrial chemical. Some limonene is prepared by extraction from plants of the mint family, a large quantity is obtained from citrus oils, which are typically 80-90% limonene, and some is obtained from pine oil. It is also synthesized chemically and finds use as a solvent and cleaning agent (in the manufacture of synthetic pine oil), as an expectorant, as a wetting and dispersing agent, as a monomer in the manufacture of various polymeric resins, as a flavorant and a precursor in the synthesis of the flavorant carvone, and as a polymerization inhibitor in storage of the tetrafluoreoethylene monomer used in the manufacture of polytetrafluoroethylene (PTFE).

Geranyl diphosphate (GPP) synthase catalyzes the first committed step of monoterpene biosynthesis by the condensation of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) to form GPP, the immediate acyclic precursor of monoterpenes. GPP is converted to (-)-4S-limonene by the catalytic action of (-)-4S-limonene synthase (cyclase). (-)-4S-limonene is converted to (-)-trans-carveol by the action of (-)-trans-carveol synthase, also referred to as limonene-6-hydroxylase. -(-)trans-carveol is converted to carvone by the action of -(-)trans-carveol dehydrogenase, also referred to as carvone synthase. GPP is also converted to the monoterpene S-linalool by the action of S-linalool synthase. Thus, GPP is the precursor of (-)-4S-limonene and its downstream metabolites (-)-trans-carveol and carvone; as well as the precursor of S-linalool. See Figure 1; Wise *et al.* (1997) In "*Comprehensive Natural Products Chemistry: Isoprenoids, Vol. 2*" (Cane, D.E., ed.), Elsevier Science, Oxford (in press); Gershenzon *et al.* (1989) *Plant Physiol. 89*:1351-1357; Pichersky *et al.* (1994) *Plant Physiol. 106*:1533-1540. Unlike the mechanistically-related prenyltransferases farnesyl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase, which produce GPP as intermediates and which are nearly ubiquitous (Ogura *et al.* (1997) In "*Dynamic Aspects of Natural Products Chemistry*" (Ogura, K. and Sankawa, U., eds.), Kodansha/Harwood Academic Publishers, Tokyo, pp. 1-23), GPP synthase is largely restricted to plant species that produce abundant quantities of monoterpenes.

Because both farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase produce only negligible levels of GPP as a free intermediate on route to FPP and GGPP (Ogura *et al.* (1997) In "*Dynamic Aspects of Natural Products Chemistry*" (Ogura, K. and Sankawa, U., eds.), Kodansha/Harwood Academic Publishers, Tokyo, pp. 1-23), it is geranyl diphosphate synthase that provides the crucial link between primary metabolism and monoterpene biosynthesis and that serves as the essential driver of monoterpene biosynthesis (Wise *et al.* (1997) In "*Comprehensive Natural Products Chemistry: Isoprenoids, Vol. 2*" (Cane, D.E., ed.), Elsevier Science, Oxford (in press)).

GPP synthase has been isolated from several plant sources, including grape, geranium, sage (Croteau *et al.* (1989) *Arch. Biochem. Biophys. 271*:524-535; Heide *et al.* (1989) *Arch. Biochem. Biophys. 273*:331-338; Suga *et al.* (1991) *Phytochemistry 30*:1757-1761; Clastre *et al.* (1993) *Plant Physiol. 102*:205-211); however, the enzyme has not been characterized in any detail, and only the enzyme from grape has been purified to homogeneity.

A cDNA encoding 4S-limonene synthase from oil glands of spearmint has been described in Colby *et al.* (1993) *J. Biol. Chem. 268(31)*: 23016-23024 and is available in the Genbank™/EMBL database and identified by the accession number L13459.

cDNAs encoding S-linalool synthase from *Clarkia Breweri* have been described in WO 97/15584, along with methods directed at using the cDNA for enhancing the flavor and smell of plants; and in Dudareva *et al.* (1996) *Plant Cell 8 (7)*:1137-1148, also available in the Genbank™/EMBL database and identified by the accession number 1491939.

To exploit recombinant methods to increase monoterpene yield in monoterpene-producing species, or to genetically engineer the monoterpene biosynthetic pathway into non-producing species, it would be highly beneficial to manipulate a GPP synthase gene. Accordingly, the invention relates to expressing GPP synthase in combination with selected monoterpene synthases such as (-)-limonene synthase, S-linalool synthase, and subsequent and related pathway enzymes for production of the corresponding monoterpene product(s).

SUMMARY OF THE INVENTION

The invention provides methods for manipulating metabolic pathways in plants, particularly those pathways that are involved in the biosynthesis of monoterpenes. Methods are directed at transforming plants, plant tissues and cells with one or more nucleotide sequences encoding the enzyme GPP synthase, and the monoterpene synthases limonene-, carveol and S-linolool synthase.

Methods are also provided for creating or enhancing resistance to insects in plants by transforming plants with GPP- and/or monoterpene synthases, to generate plants producing monoterpenes in amounts effective for resistance to insects.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the biosynthetic pathways involved in production of the monoterpenes limonene, carveol, and S-linalool.

Figure 2 schematically illustrates the plasmid construct comprising the ubiquitin promoter and a monoterpene synthesis pathway enzyme.

Figure 3 schematically illustrates the plasmid construct PHP14099 for expression of GPP- and S-linalool synthases, and production of S-linalool in plants.

Figure 4 schematically illustrates the plasmid construct PHP14100 for expression of GPP-, limonene- and carveol synthases; and production of limonene and carveol.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for manipulating metabolic pathways in plant cells; particularly those pathways related to synthesis of monoterpenes including but not limited to the monoterpenes (-)-4S-limonene (herein limonene), (-)-trans-carveol (herein carveol) and S-linalool in plants, plant cells and specific plant tissues. These monoterpenes have insecticidal and/or repellent activity against insect pests.

Accordingly, the invention provides methods useful for increasing monoterpene yield in monoterpene-producing species such as mint, and for producing monoterpenes in species which typically do not produce monoterpenes such as maize. The invention also provides methods for creating or enhancing resistance to insects in plants by transforming plants with nucleotide sequences encoding monoterpene synthesis pathway enzymes and generating transformed plants which produce effective amounts of desired monoterpenes in the plant. In this aspect, by "effective amount" is intended that amount of a monoterpene, alone or in combination with other agents, that can effect a reduction, amelioration, prevention, or elimination of a plant-insect interaction.

Thus, the methods of the invention are directed at transforming plant cells with at least one nucleotide sequence encoding a monoterpene synthesis pathway enzyme selected from GPP synthase, limonene synthase, carveol synthase, S-linalool synthase, or combinations thereof.

By "monoterpene synthase" is intended an enzyme which catalyzes a reaction having at least one monoterpene as the product of the reaction. More specifically, monoterpene synthases utilized in the methods of the invention include, but are not limited to limonene synthase, carveol synthase (limonene 6-hydroxylase) and S-linalool synthase.

By "transgenic plant" is meant any plant or plant cell that has become transformed by the introduction, stable and heritable incorporation, into the subject plant or plant cell, of either native DNA that is under the control of a promoter other than the promoter that typically drives expression of that DNA in a wild-type plant, and that has been introduced back into its host plant, or foreign DNA, i.e. DNA encoding for a protein not normally found within that plant species.

"Plantlet" refers to a plant sufficiently developed to have a shoot and a root that is asexually reproduced by cell culture.

"Explant" refers to a section or piece of tissue from any part of a plant for culturing.

The term "callus" and its plural "calli", refer to an unorganized group of cells formed in response to cutting, severing, or other injury inflicted on plant tissue. Excised pieces of plant tissue and isolated cells can be induced to form callus under the appropriate culture conditions. Callus can be maintained in

culture for a considerable time by transferring or subculturing parts of the callus to fresh medium at regular intervals. The transfer of callus to liquid medium leads to dispersion of the tissue and the formation of a plant cell suspension culture. Callus can be induced to undergo organized development to form shoots and roots.

"Embryoid" refers to a structure similar in appearance to a plant zygotic embryo.

By the term "taxon" herein is meant a unit of botanical classification of genus or lower. It thus includes genus, species, cultivar, variety, variant, and other minor taxonomic groups that lack a consistent nomenclature.

"Somatic hybrid" and "somatic hybridization" refers generally to stable combination of cellular material, be it protoplast/protoplast or protoplast/cytoplast combinations, and includes cybrids and cybridization.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as a unit of DNA replication in vivo; i.e., capable of replication under its own control.

As used herein, the term "nucleotide sequence" means a DNA or RNA sequence, and can include a cDNA, or genomic DNA, or synthetic DNA sequence, a structural gene or a fragment thereof, or an mRNA sequence, that encodes an active or functional polypeptide.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

A "promoter sequence" or a "promoter" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream

(3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at its 3' terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence is "operably linked to" or "under the control of" control sequences in a cell when RNA polymerase will bind the promoter sequence and transcribe the mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of undergoing transformation, by an exogenous DNA sequence.

A cell has been "transformed" by endogenous or exogenous DNA when such DNA has been introduced inside the cell membrane. The DNA may or may not be integrated into (covalently linked to) chromosomal DNA making up the genome of the transformed cell. In procaryotes, for example, the DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in-association

with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacterium. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). "Heterologous" DNA also refers to DNA not found within the host cell in nature. Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as these terms are used herein. "Native", "autologous" or "endogenous" DNA, as used herein, refer to DNA that is typically present in the host in nature.

The term "polypeptide" as used herein is used in its broadest sense, i.e., any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term " polypeptide" includes proteins, oligopeptides, protein fragments, analogues, muteins, fusion proteins and the like. The term also encompasses amino acid polymers as described above that include additional non-amino acid moieties. Thus, the term "polypeptide" includes glycoproteins, lipoproteins, phosphoproteins, metalloproteins, nucleoproteins, as well as other conjugated proteins. The term "polypeptide" contemplates polypeptides as defined above that are recombinantly produced, isolated from an appropriate source, or synthesized.

By the term "vector" herein is meant a DNA sequence which is able to replicate and express a foreign gene in a host cell. Typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme. Such vectors are preferably constructed to include additional structural gene sequences imparting antibiotic or herbicide resistance, which then serve as selectable markers to identify and separate transformed cells. Preferred selection agents include kanamycin, chlorosulfuron, phosphinothricin, hygromycin and methotrexate, and preferred markers are genes conferring resistance to these compounds. A cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant." A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule that is not a part of the chromosomes of the cell.

In carrying out this invention, it will be appreciated that numerous plant expression cassettes and vectors are well known in the art. By the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. It is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. In addition, the plant expression cassette preferably includes a strong promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-A recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the DNA sequence of the present invention can be inserted is the pPHI414 plasmid developed by Beach *et al.* of Pioneer Hi-Bred International, Inc., Johnston, IA. Highly preferred plant expression cassettes are designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

Methods of the invention include those for manipulating a metabolic pathway in a plant cell by transforming with nucleotide sequences for native limonene-, GPP-, carveol- and S-linalool synthase genes, by transforming with nucleotide sequences encoding amino acid sequences for the respective proteins encoded thereby, as well as fragments and variants thereof. Such native sequences are set forth in SEQ ID NOs: 1-8. SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7 set forth the nuceleotide sequences for limonene-, GPP-, carveol-, and S-linalool synthase respectively; the corresponding amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8 respectively. The methods encompass using the sequences or corresponding antisense sequences in modulating the expression of enzymes involved in monoterpene synthesis, as well as production of monoterpenes in a plant or plant cell. That is, the coding sequences are used to increase the expression of an enzyme while antisense sequences are used to decrease expression. In this aspect, blocking the expression of an enzyme within a pathway by antisense sequences can

be used to accumulate the substrates of that enzyme or to drive the pathway to another end product.

It is recognized that the methods of the invention could be used to manipulate metabolic pathways involving reactions downstream of those catalyzed by GPP- and a monoterpene synthase such as limonene-, carveol- and/or S-linalool synthase as described herein; by transforming plants with nucleotide sequences encoding GPP synthase and at least one monoterpene synthase, or antisense sequences thereof. In this aspect, the methods of the invention encompass manipulating the pathway involving production of the compounds including but not limited to (-)-carvone, (-)-trans-isopiperitinol and (-)-trans-isopiperitinone.

Antisense RNA or DNA can be utilized for the accumulation of a particular monoterpene. Alternatively, homologous plant sequences or partial plant sequences can be used. For example, an antisense carveol synthase sequence can be used to cause the accumulation of limonene. In this manner, the metabolic pathway of interest can be manipulated for the high production of any particular monoterpene of interest in the pathway.

Likewise, the pathway can be manipulated to decrease levels of a particular compound by transformation of antisense sequences which prevent the conversion of the precursor compound into the particular compound being regulated. For example, conversion of GPP to S-linalool can be blocked by antisense sequences to S-linalool synthase.

In the same manner, to increase the biosynthesis of a particular desired monoterpene, antisense constructs can be used to block the conversion of a common substrate to one monoterpene, thereby shunting the common substrate to the pathway for the desired monoterpene, while additionally blocking downstream conversion of the desired monoterpene to a further downstream metabolite. For example, an antisense sequence to S-linalool synthase can be used to shunt the common substrate GPP to the pathway for limonene biosynthesis, while additionally antisense sequence to carveol synthase can be used to block conversion of limonene to carveol.

Any means for producing a plant comprising GPP- and at least one monoterpene synthase coding sequence are encompassed by the methods of the present invention. For example, the second (or additional) gene of interest can be

used to transform a plant at the same time as the GPP synthase gene (cotransformation); the second gene can be introduced into a plant that has already been transformed with the GPP synthase gene; GPP synthase can be transformed into a plant has already been transformed with the second gene; or alternatively, transformed plants, one expressing the GPP synthase and one expressing the second gene, can be crossed to bring the genes together in the same plant. Subsequent crosses or transformations can bring additional sequences together in the plant.

The use of fragments and variants of the nucleotide and amino acid sequences are encompassed within the scope of the invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 15 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the entire nucleotide sequence encoding the monoterpene synthase proteins utilized in the methods of the invention. Fragments of the invention include antisense sequences used to decrease expression of the monoterpene synthase genes utilized in the methods of the invention. Such antisense fragments may vary in length ranging from at least about 15 nucleotides, about 50 nucleotides, about 100 nucleotides, up to and including the entire coding sequence.

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of the GPP- and carveol synthase genes. Generally, nucleotide sequence variants of the invention will have at least 70%, generally, 80%, preferably up to 90-95% sequence identity to the native nucleotide sequence.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one

or more amino acids at one or more sites in the native protein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Methods for such manipulations are generally known in the art.

For example, amino acid sequence variants of the polypeptide can be prepared by mutations in the DNA sequence encoding the native protein of interest. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods Enzymol. 154*:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

In constructing variants of the limonene-, GPP-, carveol and S-linalool synthase proteins of interest, modifications to the nucleotide sequences encoding the variants will be made such that variants continue to possess the desired activity. Obviously, any mutations made in the DNA encoding the variant protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce excessive secondary mRNA structure. See EP Patent Application Publication No. 75,444.

Thus, nucleotide sequences utilized in the methods of the invention and the proteins encoded thereby include the native forms as well as variants thereof. The variant proteins will be substantially homologous and functionally equivalent to the native proteins. A variant of a native protein is "substantially homologous" to the native protein when at least about 80-85%, more preferably at least about 90%, and most preferably at least about 95% of its amino acid sequence is identical to the amino acid sequence of the native protein. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system.

Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis *et al.* (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York; Brown, T.A. *Gene Cloning: An Introduction* (2nd Ed.) Chapman & Hall, London (1990).

Alternatively, sequence identity can be determined utilizing a number of available computer programs including but not limited to CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See http://www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection. For purposes of the present invention, comparison of nucleotide or protein sequences for determination of percent sequence identity to the sequences described herein is preferably made using the Gapped BLAST program (Version 2.0 or later) with its default parameters or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

By "functionally equivalent" is intended that the sequence of the variant defines a chain that produces a protein having substantially the same biological effect as the native protein of interest. Such functionally equivalent variants that comprise substantial sequence variations are also encompassed by the invention. Thus, for purposes of the present invention, a functionally equivalent variant of GPP synthase will catalyze the formation of GPP from dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP); that of limonene synthase will catalyze the formation of limonene from GPP; that of carveol synthase will catalyze the formation of carveol from limonene; and that of S-linalool synthase will catalyze the formation of S-linalool from GPP.

In view of the ability to transform crop plants to express various heterologous compounds, it would be desirable to transform maize plants to express monoterpene pathway enzymes and thereby produce effective amounts of the monoterpenes limonene, carveol and/or S-linalool so that by consuming the tissues of the plant an insect, such as larvae of Corn Rootworm, would consume insecticidally effective amounts of the monoterpenes; or be caused to avoid feeding on the plant.

While most gene products are peptides, a monoterpene is not a peptide or peptide derivative and is not expressed from genes in the form of a peptide or peptide derivative, but is produced enzymatically as a secondary metabolite within the cells of some plants. It is determined that the biosynthetic apparatus necessary for the production of the monoterpene limonene may not be present in many plant cells which do not produce limonene, or may not produce detectable, insecticidally effective amounts of limonene, and this appears to include maize cells. Such plant cells must be engineered with at least one enzyme which can be produced through the expression of exogenous (heterologous) genes. One such enzyme is limonene synthase, also known as limonene cyclase, which can directly synthesize limonene from geranyl pyprophosphate (GPP), which is found widely in both procaryotic and eucaryotic cells, although, as discussed below, is in some cases not produced in quantities sufficient to make insecticidally effective amounts of limonene.

Since genes which code for a monoterpene synthase can be synthesized, either directly using a DNA sequence obtained by working backwards from the known amino acid sequence of a particular monoterpene synthase and preferably

using plant-preferred codons, or by cloning from natural sources of monoterpenes, the resulting sequence can be inserted into an appropriate expression cassette, and introduced into cells of a susceptible plant species or a suitable endophytic bacterium, so that an especially preferred embodiment of this method involves inserting into the genome of the plant or bacterium a DNA sequence coding for a monoterpene synthase, in proper reading frame relative to transcription initiator and promoter sequences active in the plant or bacterium. Transcription and translation of the coding sequence under control of the regulatory sequences, can cause expression of the enzyme at levels which provide an effective amount of a monoterpene such as limonene in the tissue of the plant which are normally infested by the larvae.

As an illustration, it can be noted that Colby *et al.*, at the Keystone Symposium on Crop Improvement via Biotechnology: An International Perspective, Keystone, Colorado, USA, April 10-16, 1992, as reported in *J. Cell Biochem. Suppl. 16* F, 230 (1992), have isolated and characterized cDNA encoding limonene cyclase from spearmint. To isolate and study the gene(s) (sic) encoding limonene synthase and to produce enough of the enzyme for structural studies, they used standard methods to extract RNA from young leaves of *Mentha spicata* and constructed a cDNA library in λ ZAP XR (Stratagene) from poly (A) + RNA. They designed three degenerate oligonucleotides based on internal amino acid sequences obtained from Edman degradation of purified limonene synthase and screened 250,000 clones to identify six positive clones that hybridized to all three oligonucleotides. The resulting clones could be used in the methods of this invention which involve plant transformation. However, Colby *et al.* indicate no appreciation of the value of the enzyme in conferring resistance to insects in plants.

In certain plants, including maize, at least one additional gene encoding GPP synthase is required for generation of plants with resistance to insects. Due to the fact that natural levels of GPP are low in such plants, there may be inadequate amounts of GPP for limonene production in these plants when they are transformed solely with the limonene synthase gene. In contrast, GPP levels in other plant species, such as spearmint, is adequate for limonene production. In such species, some GPP is used to generate limonene, some to generate other metabolites. Because GPP is derived from a pathway that is common among plant

species, the introduction of the GPP synthase gene and at least one monoterpene synthase gene such as limonene synthase into plant species lacking GPP can generate transgenic plants capable of producing GPP, and a desired monoterpene at levels effective to confer resistance to insects.

In this manner, the invention encompasses transforming plants with nucleotide sequences encoding GPP synthase and limonene synthase for the generation of plants producing effective amounts of limonene; with nucleotide sequences encoding GPP synthase, limonene synthase and carveol synthase for the generation of plants producing effective amounts of carveol; and with nucleotide sequences encoding GPP synthase and S-linalool synthase for the generation of plants producing effective amounts of S-linalool.

In one embodiment, the plant which can be benefitted by this invention is preferably a plant susceptible to infestation and damage by the larvae of the genus Diabrotica or whose harvested material is subject to attack by larvae of that insect. A prime example is corn (*Zea mays*). Thus the methods of this invention are readily applicable via conventional techniques to numerous plant species, particularly those susceptible to *Diabrotica spp.*, including, without limitation, species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manicot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hemerocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browallia*, *Glycine*, *Lolium*, *Triticum*, and *Datura*.

Thus, the methods of the invention are useful to transform plants and create or enhance resistance to insects in plants. By resistance to insects is intended that the plant-insect interaction, preferably a plant-insect pest interaction, is reduced, ameliorated, prevented, or eliminated.

Insect pests include but are not limited to insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera. Insect pests of the invention for the major crops include: Maize: *Ostrinia nubilalis*,

European corn borer; *Agrotis ipsilon*, black cutworm; *Helicoverpa zea*, corn earworm; *Spodoptera frugiperda*, fall armyworm; *Diatraea grandiosella*, southwestern corn borer; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Diatraea saccharalis*, surgarcane borer; *Diabrotica virgifera*, western corn rootworm; *Diabrotica longicornis barberi*, northern corn rootworm; *Diabrotica undecimpunctata howardi*, southern corn rootworm; *Melanotus spp.*, wireworms; *Cyclocephala borealis*, northern masked chafer (white grub); *Cyclocephala immaculata*, southern masked chafer (white grub); *Popillia japonica*, Japanese beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Anuraphis maidiradicis*, corn root aphid; *Blissus leucopterus leucopterus*, chinch bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Hylemya platura*, seedcorn maggot; *Agromyza parvicornis*, corn blot leafminer; *Anaphothrips obscrurus*, grass thrips; *Solenopsis milesta*, thief ant; *Tetranychus urticae*, twospotted spider mite; Sorghum: *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Feltia subterranea*, granulate cutworm; *Phyllophaga crinita*, white grub; *Eleodes*, *Conoderus*, and *Aeolus spp.*, wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*; corn leaf aphid; *Sipha flava*, yellow sugarcane aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Wheat: *Pseudaletia unipunctata*, army worm; *Spodoptera frugiperda*, fall armyworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis orthogonia*, western cutworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella*

fusca, tobacco thrips; *Cephus cinctus*, wheat stem sawfly; *Aceria tulipae*, wheat curl mite; Sunflower: *Suleima helianthana*, sunflower bud moth; *Homoeosoma electellum*, sunflower moth; *zygogramma exclamationis*, sunflower beetle; *Bothyrus gibbosus*, carrot beetle; *Neolasioptera murtfeldtiana*, sunflower seed midge; Cotton: *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Spodoptera exigua*, beet armyworm; *Pectinophora gossypiella*, pink bollworm; *Anthonomus grandis*, boll weevil; *Aphis gossypii*, cotton aphid; *Pseudatomoscelis seriatus*, cotton fleahopper; *Trialeurodes abutilonea*, bandedwinged whitefly; *Lygus lineolaris*, tarnished plant bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Thrips tabaci*, onion thrips; *Franklinkiella fusca*, tobacco thrips; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Rice: *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Colaspis brunnea*, grape colaspis; *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephotettix nigropictus*, rice leafhopper; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; Soybean: *Pseudoplusia includens*, soybean looper; *Anticarsia gemmatalis*, velvetbean caterpillar; *Plathypena scabra*, green cloverworm; *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Spodoptera exigua*, beet armyworm; *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Epilachna varivestis*, Mexican bean beetle; *Myzus persicae*, green peach aphid; *Empoasca fabae*, potato leafhopper; *Acrosternum hilare*, green stink bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Hylemya platura*, seedcorn maggot; *Sericothrips variabilis*, soybean thrips; *Thrips tabaci*, onion thrips; *Tetranychus turkestani*, strawberry spider mite; *Tetranychus urticae*, twospotted spider mite; Barley: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Schizaphis gramimum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servus*, brown stink bug; *Delia platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Petrobia latens*, brown wheat mite; Oil Seed Rape: *Brevicoryne brassicae*, cabbage aphid; *Phyllotreta cruciferae*, Flea beetle; *Mamestra configurata*, Bertha armyworm; *Plutella xylostella*, Diamond-back moth; *Delia* ssp., Root maggots.

Preferred plants that are to be transformed according to the methods of this invention are cereal crops, including maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed and soybean, fiber crops, such as cotton, fruit crops, such as melons, and vegetable crops, including onion, pepper, tomato, cucumber, squash, carrot, crucifer (cabbage, broccoli, cauliflower), eggplant, spinach, potato and lettuce.

While compounds other than monoterpenes have some effective insecticidal activity at high concentrations in pure form, plant cell expression at such high concentrations is either not possible in a living plant cell system, or is not feasible if the commercially useful characteristics of the plant are to be preserved in terms of production of oils, starches, fibers, or other materials. Monoterpenes, on the other hand, are not directly expressed as the gene product, and the peptide or peptides which is or are expressed in the methods of this invention is an enzyme which can catalyze the synthesis of large amounts of a monoterpene(s) in the tissues of the transformed plant (e.g. limonene synthase), and in instances in which it is required, an enzyme which can catalyze the synthesis of large amounts of substrate for a downstream monoterpene synthase (e.g. GPP synthase).

The genes utilized in the invention, including GPP-, carveol, limonene-, and S-linalool synthase genes can be optimized for enhanced expression in plants of interest. See, for example, EPA0359472; WO91/16432; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:3324-3328; and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498. In this manner, the genes can be synthesized utilizing plant-preferred condons. See, for example, Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, the disclosure of which is incorporated herein by reference. In this manner, synthetic genes can also be made based on the distribution of codons a particular host uses for a particular amino acid. Thus, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used.

The methods of the invention encompass utilizing naturally occurring nucleotide sequences encoding GPP synthase, and the monoterpene synthases limonene-, carveol- and S-linalool synthases; or utilizing synthetically derived

sequences encoding these proteins. The naturally occurring nucleotide sequences utilized in the methods of the invention are set forth in Figures 6, 8, 10 and 12.

The methods also encompass utilizing nucleotide sequences isolated from various organisms including plants by hybridization with partial sequences obtained from the natural sequences as set forth above. Conditions that will permit other DNA sequences to hybridize to the DNA sequences set forth herein can be determined in accordance with techniques generally known in the art. For example, hybridization of such sequences may be carried out under conditions of reduced stringency, medium stringency, or high stringency conditions (*e.g.*, conditions represented by a wash stringency of 35-40% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 37°C; conditions represented by a wash stringency of 40-45% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 42°C; and conditions represented by a wash stringency of 50% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 42°C, respectively. *See* Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). The methods further encompass utilizing nucleotide sequences isolated from various organisms including plants by other well known methods such as PCR using the natural sequences as set forth above.

The methods of the invention comprise utilizing expression cassettes with constitutive or tissue-specific promoters. Promoters that may be used in the expression cassettes include without limitation nos, ocs, phaseolin, FMV and other promoters isolated from the DNA of plants or other sources, both natural and synthetic.

Constitutive promoters would provide a constant production of the enzymes GPP-, limonene-, carveol and/or S-linalool synthase and thereby the corresponding monoterpene(s). Such constitutive promoters include, for example, the core promoter of the Rsyn7 (U.S. Patent Application Serial No. 08/661,601), the core CaMV 35S promoter (Odell *et al.* (1985) *Nature 313*:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell 2*:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol. 12*:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol. 18*:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet. 81*:581-588); MAS (Velten *et al.* (1984) *EMBO J. 3*:2723-2730); ALS promoter (U.S. Patent Application Serial

No. 08/409,297), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

An efficient plant promoter that may be used is an overproducing plant promoter. Overproducing plant promoters that may be used in this invention include the promoter of the small sub-unit (ss) of the ribulose-1,5-bisphosphate carboxylase from soybean (Berry-Lowe *et al.* (1982) *J. Mol. and App. Gen. 1*:483-498, and the promoter of the chlorophyll a-b binding protein. However, these two promoters are known to be light-induced in eukaryotic plant cells (see, for example, *Genetic Engineering of Plants, An Agricultural Perspective*, Cashmore, Pelham, New York, 1983, pp. 29-38, G. Coruzzi *et al.* (1983) *J. Biol. Chem. 258*:1399 and P. Dunsmuir *et al.* (1983) *J. Mol. and App. Gen. 2*:285 and may be less desirable when root expression is desired.

A tissue-specific promoter (or promoters) can be used in any instance where it may be desirable to localize production of the desired monoterpene to an insect-infested tissue or to a tissue which is efficient in production of a desired enzyme. The utilization of tissue-specific promoters would increase or decrease the expression of monoterpene synthases and production of the corresponding monoterpenes in specific tissues of the plant. It is recognized that in manipulating the level of monoterpene production as such, it may be desirable to increase or decrease the levels of such molecules in a particular tissue, since tolerance of various tissues to increased expression of specific monoterpenes may vary. Thus, it may be desirable to increase expression in selected tissues, or at varying levels in different tissues by the use of tissue-specific promoters.

Particular tissue-specific promoters of interest includes root-preferred promoters. The utilization of such promoters would provide a method of selectively creating or enhancing resistance to insects, and/or manipulating levels of monoterpene molecules in the root. Since corn rootworm attack roots, root-specific promoters are especially preferred for the control of corn rootworm, while minimizing limonene production in the agronomically valuable parts of the plant. Such selectivity could also be particularly desirable in plants in which the root constitutes the food crop, including, but not limited to carrot, potato, radish, and the like.

Root specific promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. For example, Hire, *et al.* (1992) *Plant Mol. Biology, 20(2)*: 207-218, describe a root-specific glutamine synthetase gene from soybean. Keller and Baumgartner, (1991) *The Plant Cell, 3(10)*:1051-1061, describe a root-specific control element in the GRP 1.8 gene of French bean. Sanger *et al.* (1990) *Plant Mol. Biology, 14(3)*: 433-443, discuss the root-specific promoter of the Mannopine Synthase (MAS) gene of *Agrobacterium tumefaciens*. Miao *et al.* (1991) *The Plant Cell, 3(1)*:11-22, describe a full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean. Bogusz *et al.* (1990) *The Plant Cell, 2(7)*:633-641, discusses two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa*. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) *Plant Science (Limerick) 79(1)*:69-76, describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes*. They concluded that enhancer and tissue-specific DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) *EMBO Journal, 8(2)*:343-350, used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene was root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene. The TR1' gene, fused to NPTII, (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster H *et al.* (1995) *Plant Mol. Biol. 29(4)*:759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol. 25(4)*:681-691. See also U.S. Patent Nos. 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

Other tissue-specific promoters include those described in Yamamoto *et al.* (1997) *Plant J. 12(2)*:255-265; Kawamata *et al.* (1997) *Plant Cell Physiol. 38(7)*:792-803; Hansen *et al.* (1997) *Mol. Gen. Genet. 254(3)*:337-343; Russell *et*

al. (1997) *Transgenic Res. 6(2)*:157-168; Rinehart *et al.* (1996) *Plant Physiol. 112(3)*:1331-1341; Van Camp *et al.* (1996) *Plant Physiol. 112(2)*:525-535; Canevascini *et al.* (1996) *Plant Physiol. 112(2)*:513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol. 35(5)*:773-778; Lam (1994) *Results Probl. Cell Differ. 20*:181-196; Orozco *et al.* (1993) *Plant Mol. Biol. 23(6)*:1129-1138; Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90(20)*:9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J. 4(3)*:495-505.

Seed-preferred promoters includes both seed-specific promoters (those promoters active during seed development) as well as seed-germinating promoters (those promoters active during seed germination). Such promoters include Cim1 (cytokinin-induced message); cZ19B1 (maize 19KDa zein); milps (myo-inositol-1-phosphate synthase); celA (cellulose synthase); end1 (*Hordeum verlgase* mRNA clone END1); and imp3 (myo-inositol monophosphate-3). For dicots, particular promoters include phaseolin, napin, β-conglycinin, soybean lectin, and the like. For monocots, particular promoters include maize 15Kd zein, 22KD zein, 27kD zein, waxy, shrunken 1, shrunken 2, globulin 1, etc.

In native tissues which synthesize monoterpenes, GPP-, limonene- and S-linalool synthases are targeted to plastids via plastid targeting sequences (transit peptides) typically contained at the N-terminus of the preprotein forms of the enzymes. The targeting sequences are then cleaved to release the mature enzymes in plastids. Such transit peptides can be identified in the primary amino acid sequences of the preproteins by those ordinarily skilled in the art. For example, see Colby *et al.* (1993) *J. Biol. Chem. 268(31)*:23016-23024, for the transit peptide sequence of limonene synthase. Native carveol synthase is associated with the endoplasmic reticulum, while native carveol dehydrogenase could be cytoplasmic.

Furthermore, the substrates of GPP synthase, IPP (IDP) and DMAPP (DMADP) can be found in plastids or in the cytoplasm (Bohlmann *et al.* (1998) P*roc. Natl. Acad. Sci. USA 95*:4126-4133). In plastids, these substrates are most likely synthesized via the glyceraldehyde-3-phosphate/pyruvate (Rohmer) pathway, and in the cytosol via the mevalonate pathway in the cytosol or the mevalonate pathway.

Thus, for manipulating a metabolic pathway involving monoterpene production, it would be beneficial to target GPP-, limonene-, and S-linalool

synthase to plastids; and carveol synthase and dehydrogenase to the endoplasmic reticulum (ER). This targeting could be achieved by use of the native targeting sequences contained in the sequences of the native proteins, or by addition or exchange of heterologous subcellular targeting signals. Alternatively, the enzymes utilized in the methods of the invention could be directed to the cytoplasm by deletion of the plastid and/or ER targeting signals. Methods for deletion, exchange and addition of nucleotide sequences are well known in the art, and can be readily used for manipulation of nucleotide segments encoding targeting signals of interest as described herein.

Heterologous sequences which can be used to target the desired enzymes of the invention to plastids include chloroplast targeting sequences. Chloroplast targeting sequences are known in the art and include the chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco), (de Castro Silva Filho *et al.* (1996) *Plant Mol. Biol. 30*:769-780; Schnell, *et al.* (1991) *J. Biol. Chem. 266(5)*:3335-3342); 5-(enolpyruvyl)shikimate-3-phosphate synthase (EPSPS) (Archer *et al.* (1990) *J. Bioenerg. Biomemb. 22(6)*:789-810); tryptophan synthase (Zhao *et al.* (1995) *J.Biol. Chem. 270(11)*:6081-6087); plastocyanin (Lawrence *et al.* (1997) *J. Biol. Chem. 272(33)*:20357-20363); chorismate synthase (Schmidt *et al.* (1993) *J. Biol. Chem. 268(36)*:27477-27457); and the light harvesting chlorophyll a/b binding protein (LHBP) (Lamppa *et al.* (1988) *J. Biol. Chem. 263*:14996-14999). See also Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep. 9*:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun. 196*:1414-1421; and Shah *et al.* (1986) *Science 233*:478-481.

Likewise, methods for transformation of chloroplasts are known in the art. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:8526-8530; Svab and Maliga (1993) *Proc. Natl Acad. Sci. USA 90*:913-917; Staub and Maliga (1993) *Embo J. 12*:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-specific expression of a nuclear-encoded and plastid-directed RNA polymerase.

Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:7301-7305.

The nucleotide sequences utilized in methods of the invention can be introduced into any plant. The sequences to be introduced may be used in expression cassettes for expression in any plant of interest where expression in the plant is necessary for transcription.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of the enzymes of the invention in the plant or plant cell. Thus, the phenotype of the plant or plant cell is altered.

Where expression cassettes are needed, such expression cassettes will comprise a transcriptional initiation region linked to the coding sequence or antisense sequence of the nucleotide of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By foreign is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

The transcriptional cassette will include in the 5'-to-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet. 262*:141-144; Proudfoot (1991) *Cell 64*:671-674; Sanfacon *et al.* (1991) *Genes Dev. 5*:141-149; Mogen *et al.* (1990) *Plant Cell. 2*:1261-1272; Munroe *et al.* (1990) *Gene 91*:151-

158; Ballas *et al.* (1989) *Nucleic Acids Res. 17*:7891-7903; Joshi *et al.* (1987) *Nucleic Acids Res. 15*:9627-9639.

The methods of the invention encompass use of expression cassettes for expression of nucleotide sequences encoding GPP- and monoterpene synthases in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to the sequence of interest. The cassette may additionally contain at least one additional sequence to be cotransformed into the organism. Alternatively, the additional sequence(s) can be provided on another expression cassette.

Where appropriate, sequences utilized in the methods of the invention and additional gene(s) may be optimized for increased expression in the transformed plant. That is, these nucleotide sequences can be synthesized using plant preferred codons for improved expression. Methods are available in the art for synthesizing plant preferred genes. See, for example, U.S. Patent Nos. 5,380,831, 5,436, 391, and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences which may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Nat. Acad. Sci. USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986)); MDMV leader (Maize Dwarf Mosaic Virus) (*Virology 154*:9-20); human immunoglobulin heavy-chain binding protein (BiP) (Macejak and Sarnow (1991) *Nature 353*:90-94); untranslated leader from the coat protein mRNA of alfalfa

mosaic virus (AMV RNA 4) (Jobling and Gehrke (1987) *Nature 325*:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) *Molecular Biology of RNA*, pages 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology 81*:382-385). See also Della-Cioppa *et al.* (1987) *Plant Physiology 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, *e.g.*, transitions and transversions, may be involved.

The methods of the invention can be used in transforming or transfecting any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, *i.e.* monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium*-mediated transformation (Hinchee *et al.* (1988) *Biotechnology 6*:915-921), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes et al. (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see Weissinger *et al.* (1988) *Annual Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.*

27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet. 96*:319-324 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Tomes *et al.* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (1995) (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839 (maize); Hooydaas-Van Slogteren and Hooykaas (1984) *Nature (London) 311*:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports 9*:415-418; and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference. Thus, the invention provides a method for imparting insect resistance in *Agrobacterium tumefaciens*-susceptible plants in which the expression cassette is introduced into the cells by infecting the cells with *Agrobacterium tumefaciens*, a plasmid of which has been modified to include a plant expression cassette which expresses GPP- and/or a monoterpene synthase in the manner of this invention.

The modified plant may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell. Reports 5*:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting plants or hybrid plants the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

In a preferred embodiment, the invention provides methods of imparting resistance to *Diabrotica spp.* to plants of a susceptible taxon, comprising the steps of:

(a) culturing cells or tissues from at least one plant from the taxon,

(b) introducing into the cells of the cell or tissue culture at least one copy of an expression cassette comprising a structural gene coding for at least one monoterpene synthase or both a monoterpene synthase and GPP synthase, operably linked to a promoter that drives expression in a plant cell, and

(c) regenerating whole plants from the cell or tissue culture with resistance to insects. Once whole plants have been obtained, they can be sexually or clonally reproduced in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

Alternatively, once a single transformed plant has been obtained by the foregoing recombinant DNA method, conventional plant breeding methods can be used to transfer the structural gene or genes and associated regulatory sequences via crossing and backcrossing. Such intermediate methods will comprise the further steps of:

(a) sexually crossing the plant having resistance to insects with a plant from the taxon susceptible to insects;

(b) recovering reproductive material from the progeny of the cross; and

(c) growing plants having resistance to insects from the reproductive material. Where desirable or necessary, the agronomic characteristics of the susceptible taxon can be substantially preserved by expanding this method to include the further steps of repetitively:

(a) backcrossing the progeny having resistance to insects with plants from taxon susceptible to insects; and

(b) selecting for the expression of resistance to insects (or an associated marker gene) or monoterpene production among the progeny of the backcross, until the desired percentage of the characteristics of the susceptible taxon are present in the progeny along with the gene imparting resistance to insects.

Insect pests of harvested material, including those of stored grain, can also be targets for the methods of this invention. In view of this, the invention also

provides methods for creating or enhancing resistance to insect pests in harvested materials and products obtained from harvested materials, by expressing at least one monotermene synthase and/or GPP synthase in the plant such that effective amounts of the desired monoterpene is produced in the harvested material and products obtained from such material.

The following examples are offered by way of illustration and not by way of limitation. The following description further exemplifies the compositions of this invention and the methods of making and using them. However, it will be understood that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

EXAMPLES 1-6

Insect larvae inhibition and toxicity assays for
Southern Corn Rootworm (SCR) and European Corn Borer (ECB)

Bioassay diets were prepared as described in Czapla and Lang in "Effect of Plant Lectins on the Larval Development of European Corn Borer (Lepidoptera: Pyralidae) and Southern Corn Rootworm (Coleoptera: Chrysomelidae)", *J. Econ. Entomol. 83*:2480-85 (1990), except that low melting temperature agarose replaced the regular agarose so that the diets could be chilled to 37°C prior to the addition of limonene (one assay used the regular agarose diet).

Results were as follows. In Examples 1-4, the test larvae were Southern Corn Rootworm. In Examples 5-6, the test larvae were European Corn Borer. The results of each experiment represent the average from 16-32 insects. All limonene concentrations (ppm) are by weight. The SCR data indicate that limonene is effective against the larvae, but when limonene was used in the same protocol against ECB, little or no effect was seen.

$$\% \text{ Corrected Mortality} = 100 \times \frac{(\text{mortality of treated} - \text{control})}{(100 - \text{control})}$$

$$\% \text{ Wt. Reduction} = 100 \times \frac{(\text{control weight} - \text{treated weight})}{(\text{control wt})}$$

Example:	1		2	
Limonene _ppm	% Corr. Mortal.	% weight Reduction	% Corr. Mortal.	% weight Reduction
10,000	57	64	80	86
1,000	49	52	19	30
100	57	52	15	6

Example:	3		4	
Limonene _ppm	% Corr. Mortal.	% weight Reduction	% Corr. Mortal.	% weight Reduction
10,000	96	64	44	0
1,000	7	0	22	0
100	26	0	19	0

Average SCR Results:

Limonene _ppm	% Corr. Mortal.	% weight Reduction
10,000	69	54
1,000	24	21
100	29	15

5

Example:	5		6	
Limonene _ppm	% Corr. Mortal.	% weight Reduction	% Corr. Mortal.	% weight Reduction
10,000	0	28	0	0
1,000	0	16	0	0
100	0	1	0	7

Average ECB Results:

Limonene ppm	% Corr. Mortal.	% weight Reduction
10,000	0	14
1,000	0	8
100	0	4

EXAMPLE 7

Maize callus cultures were transformed by microprojectile bombardment using plasmids containing a cloned gene coding for the limonene synthase (limonene cyclase) enzyme driven by a ubiquitin promoter and a ubiquitin intron and followed downstream by a PIN-II terminator. Whole, fertile plants were regenerated from the transformed callus and analyzed for limonene synthase and limonene. Representative results from one series were as follows:

Clone #	Callus LS[1]	Callus Limonene2	Plant	Plant LS	Plant Limonene
C6	22539	ND	3	1950	ND
			7	2000	ND
C19	9900	ND	6	2100	ND
C3	2550	ND	3	1900	ND
			4	1650	ND

In other experiments, transgenic maize plants, and tissues that exhibited high expression of the limonene synthase protein were produced. The enzyme was extracted from transformed plants and tissues and allowed to react with tritium-labeled geranyl pyrophosphate (GPP). The extracted enzyme converted GPP to limonene, showing that a functional enzyme was being produced. In addition, western blots were done and confirmed the presence of LS protein in transgenic

1 LS = Limonene Synthase

2 ND = not detected above 0.5 ng/g fresh wt.

tissues but not in negative control tissues. Seed was collected from the transformed plants. In sum, whole fertile, transformed plants have been produced that express active limonene cyclase (synthase) according to the teaching of the above-identified application.

Western blots were also performed on chloroplasts isolated from plants grown from T1 and T2 seed which expressed the mint limonene synthase gene (Figure 6) in leaf tissue. Thus, the mint limonene synthase was properly targeted to maize plastids and the protein was processed to the correct size, indicating that the mint limonene synthase plastid targeting sequence can be used to target proteins to maize plastids.

EXAMPLE 8

In view of the results set forth in Example 7, demonstrating expression of active limonene synthase in whole, fertile, transformed plants, larvicidally effective amounts of limonene can be produced in such transgenic plants where sufficient substrate is present for the limonene synthase enzyme to act on. Accordingly, maize callus cultures are transformed by art recognized microprojectile bombardment methods using plasmids containing genes coding for both the limonene synthase enzyme and the GPP synthase enzyme, driven, for example, by one or more promoters (a ubiquitin promoter, for example) and followed downstream by, for example, a PIN-II terminator. Whole, fertile plants are regenerated from the transformed callus and analyzed for presence of and/or activity of both enzymes, and are also analyzed for the presence of limonene.

Alternatively, callus is generated from transgenic plants that contain and express the limonene synthase transgene, and such callus cultures are transformed as described above, except that such callus is transformed using plasmids containing a gene coding for the GPP synthase protein. The whole, fertile, transgenic plants regenerated from such transformed callus produce larvicidally effective amounts of limonene.

EXAMPLE 9

Average minimum levels of various monoterpenes required for 100% mortality of Western corn rootworm were determined by the methods described in Examples 1-6 as follows:

Limonene-----200 ppm

S-linalool---50 ppm

carveol------25 ppm

EXAMPLE 10: Construction of Expression Vectors

Nucleotide sequences encoding limonene, GPP-, carveol and S-linalool synthase, as set forth in SEQ ID NOs: 1, 3, 5, and 7 respectively, are cloned into a plasmid vector, such as that shown in Figure 2, in the sense orientation so that they are under the transcriptional control of the ubiquitin promoter. A selectable marker gene may reside on this plasmid or may be introduced as part of a second plasmid. The transformation construct is then available for introduction into maize embryos by bombardment methods as described in Example 12.

EXAMPLE 11: Construction of Expression Vectors

Nucleotide sequences encoding GPP- and S-linalool synthase as described in EXAMPLE 10 were cloned in the sense orientation into an expression vector as shown in in Figure 3(PHP14099); such that both coding sequences are under the transcriptional control of the ubiquitin promoter. The plasmid contains the selectable marker gene PAT.

Nucleotide sequences encoding GPP-, limonene- and carveol synthases as described in EXAMPLE 10 were cloned in the sense orientation into an expression vector as shown in Figure 4(PHP14100); such that all three coding sequences are under the transcriptional control of the ubiquitin promoter. The plasmid contains the selectable marker gene PAT.

The transformation constructs PHP14099 and PHP14100 are available for introduction into maize embryos by bombardment methods as described in Example 12.

EXAMPLE 12: Transformation and Regeneration of Maize Callus

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the disclosed GPP- or monoterpene synthase gene operably linked to the ubiquitin promoter plus a plasmid containing the selectable marker gene PAT (Wohlleben et al. (1988) Gene 70:25-37) that confers resistance to the herbicide Bialophos. Transformation is performed as follows. All media recipes are in the Appendix.

Preparation of Target Tissue

The ears are surface sterilized in 30% Chlorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate. These are cultured on 560 L medium for 4 days prior to bombardment, in the dark. The day of bombardment, the embryos are transferred to 560 Y medium for 4 hours, arranged within the 2.5-cm target zone.

Preparation of DNA

A plasmid vector comprising the disclosed GPP- or monoterpene synthase operably linked to the ubiquitin promoter is constructed. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten pellets using a CaCl2 precipitation procedure as follows:

100 μl prepared tungsten particles in water
10 μl (1 μg) DNA in TrisEDTA buffer (1 μg total)
100 μl 2.5 M $CaCl_2$
10 μl 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is

removed, and 105 µl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialophos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are sampled for PCR and activity of the acetyl-CoA synthetase gene of interest. Positive lines are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored for expression of the GPP-synthase or monoterpene synthase gene of interest.

APPENDIX

Ingredient	Amount	Unit
D-I H2O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolystate (acid)	0.300	g
Sucrose	20.000	g
Glucose	0.600	g
2,4-D 0.5 mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1 mg/ml #	1.200	ml
Silver Nitrate 2 mg/ml #	1.700	ml

Directions:

@= Add after bringing up to volume

#= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

##= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H_2O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH_2PO_4; 1.850 g of Magnesium Sulfate 7-H_2O, $MgSO_4$, $7H_2O$; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H_2O. Bring up to volume with D-I H_2O.

Total Volume (L) = 1.00

604 A

Ingredient	Amount	Unit
D-I H_2O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolysate (acid)	0.300	g
Sucrose	20.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	1.700	ml
Bialaphos 1 mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H_2O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH_2PO_4; 1.850 g of Magnesium Sulfate 7-H_2O, $MgSO_4$,

$7H_2O$; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H_2O. Bring up to volume with D-I H_2O.

Total Volume (L) = 1.00

605 J

Ingredient	Amount	Unit
D-I H_2O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
Sucrose	20.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	0.425	ml
Bialaphos 1 mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H_2O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH_2PO_4; 1.850 g of Magnesium Sulfate 7-H_2O, $MgSO_4$, $7H_2O$; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H_2O. Bring up to volume with D-I H_2O.

Total Volume (L) = 1.00

604 S

Ingredient	Amount	Unit
D-I H_2O	800.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolysate (acid)	0.300	g
Sucrose	120.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	1.700	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H_2O. Then dissolve 4.629 of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH_2PO_4; 1.850 g of Magnesium Sulfate 7-H_2O, $MgSO_4$, $7H_2O$; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in 950.000 ml of polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into 950.000 ml of D-I H_2O. Bring up to volume with D-I H_2O.

Total Volume (L) = 1.00

272 V

Ingredient	Amount	Unit
D-I H_2O	950.000	ml
MS Salts (GIBCO 11117-074)	4.300	g
Myo-Inositol	0.100	g
MS Vitamins Stock Solution ##	5.000	ml
Sucrose	40.000	g
Bacto-Agar @	6.000	g

Directions:

@ = Add after bringing up to volume

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H_2O in sequence. Bring up to volume with polished D-I H_2O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month, unless contamination or precipitation occur, then make fresh stock.

Total Volume (L) = 1.00

288 J

Ingredient	Amount	Unit
D-I H_2O	950.000	ml
MS Salts	4.300	g
Myo-Inositol	0.100	g
MS Vitamins Stock Solution ##	5.000	ml
Zeatin .5mg/ml	1.000	ml
Sucrose	60.000	g
Gelrite @	3.000	g
Indole Acetic Acid 0.5 mg/ml #	2.000	ml
.1mM Absissic Acid	1.000	ml
Bialaphos 1mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

Dissolve ingredients in polished D-I H_2O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H_2O after adjusting pH

Sterilize and cool to 60°C.

Add 3.5g/L of Gelrite for cell biology.

= Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H_2O in sequence. Bring up to volume with polished D-I H2O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month, unless contamination or precipitation occur, then make fresh stock.

Total Volume (L) = 1.00

560 L

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	20.000	g
2, 4-D 0.5mg/ml	2.000	ml
L-Proline	2.880	g
Gelrite @	2.000	g
Silver Nitrate 2mg/ml #	4.250	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H_2O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H_2O

Sterilize and cool to room temp.

Total Volume (L) = 1.00

560 R

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	1.000	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	30.000	g
2, 4-D 0.5mg/ml	4.000	ml
Gelrite @	3.000	g
Silver Nitrate 2mg/ml #	0.425	ml
Bialaphos 1mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H_2O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H_2O

Sterilize and cool to room temp.

Total Volume (L) = 1.00

560 Y

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	1.000	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	120.000	g
2,4-D 0.5mg/ml	2.000	ml
L-Proline	2.880	g
Gelrite @	2.000	g
Silver Nitrate 2mg/ml #	4.250	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H2O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H_2O

Sterilize and cool to room temp.

** Autoclave less time because of increased sucrose**

Total Volume (L) = 1.00

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A method for manipulating a metabolic pathway in a plant cell, said method comprising transforming a plant cell with at least one nucleotide sequence operably linked to a promoter that drives expression in a plant cell, wherein said nucleotide sequence is selected from the group consisting of:

a) a nucleotide sequence encoding a limonene synthase protein,

b) a nucleotide sequence encoding a GPP synthase protein,

c) a nucleotide sequence encoding a carveol synthase protein,

d) a nucleotide sequence encoding a S-linalool synthase protein,

e) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,

f) a nucleotide sequence encoding a GPP synthase protein having the amino acid sequence set forth in SEQ ID NO:4,

g) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,

h) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,

i) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,

j) a nucleotide sequence encoding a GPP synthase protein as as set forth in SEQ ID NO:3,

k) a nucleotide sequence encoding a carveol synthase protein as forth in SEQ ID NO:5,

l) a nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,

m) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h, i, j, k or l.

2. The method of claim 1 wherein said plant cell is monocotyledonous.

3. The method of claim 2 wherein said monocot cell is a corn, wheat, rice, oat, rye or sorghum cell.

4. The method of claim 1 wherein said promoter is a constitutive promoter.

5. The method of claim 4 wherein said constitutive promoter is a ubiquitin promoter.

6. The method of claim 1 wherein said promoter is a tissue-specific promoter.

7. 7.The method of claim 6 wherein said tissue-specific promoter is a root-specific promoter.

8. A method for manipulating a metabolic pathway in a plant cell, said method comprising transforming a plant cell with a first nucleotide sequence encoding a GPP synthase protein, and at least one nucleotide sequence which is selected from the group consisting of:

a) a nucleotide sequence encoding a limonene synthase protein,

b) a nucleotide sequence encoding a carveol synthase protein,

c) a nucleotide sequence encoding a S-linalool synthase protein,

d) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,

e) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,

f) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,

g) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,

h) A nucleotide sequence encoding a carveol synthase protein as forth in SEQ ID NO:5,

i) A nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,

j) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h or i; and

wherein said first and said second nucleotide sequence are operably linked to a promoter that drives expression in a plant cell.

9. The method of claim 8, wherein said plant cell is co-transformed.

10. A method for creating or enhancing resistance to insects in a plant, said method comprising manipulating a metabolic pathway in a plant cell according to the method of claim 8, wherein

said selected nucleotide sequence consists of a second nucleotide sequence encoding a limonene synthase protein, and

further comprising regenerating stably transformed plants producing effective amounts of limonene.

11. A method for creating or enhancing resistance to insects in a plant, said method comprising manipulating a metabolic pathway in a plant cell according to the method of claim 8, wherein

said selected nucleotide sequences consist of a second nucleotide sequence encoding a limonene synthase protein and

a third nucleotide sequence encoding a carveol synthase protein; and

further comprising regenerating stably transformed plants producing effective amounts of carveol.

12. A method for creating or enhancing resistance to insects in a plant, said method comprising manipulating a metabolic pathway in a plant cell according to the method of claim 8, wherein

said selected nucleotide sequence consists of a second nucleotide sequence encoding a S-linalool synthase protein, and

further comprising regenerating stably transformed plants producing effective amounts of S-linalool.

13. The method of any of the claims 10, 11, or 12 wherein said plant is monocotyledonous.

14. The method of claim 13 wherein said monocot plant is a corn plant (Z. mays L.).

15. The method of claim 14 wherein said insect is larvae of *Diabrotica spp.*

16. A method of producing limonene synthase and GPP synthase in a plant cell, said method comprising transforming a first plant cell with a nucleotide sequence encoding a limonene synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a second plant cell with a nucleotide sequence encoding a GPP synthase protein operably linked to a promoter that drives expression in a plant cell,

regenerating a first fertile transgenic plant from said first plant cell,

regenerating a second fertile transgenic plant from said second plant cell,

crossing said first and second fertile transgenic plants; and

recovering progeny from said cross which express limonene synthase and GPP synthase.

17. A method of producing limonene synthase, GPP synthase, and carveol synthase in a plant cell, said method comprising

transforming a first plant cell with a nucleotide sequence encoding a limonene synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a second plant cell with a nucleotide sequence encoding a GPP synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a third plant cell with a nucleotide sequence encoding a carveol synthase protein operably linked to a promoter that drives expression in a plant cell,

producing a first fertile transgenic plant from said first plant cell,

producing a second fertile transgenic plant from said second plant cell,

producing a third fertile transgenic plant from said third plant cell,

performing a first cross between said first and second fertile transgenic plants,

recovering progeny from said first cross,

performing a second cross between said third fertile transgenic plant and said progeny of said first cross; and

recovering progeny from said second cross which express limonene synthase, GPP synthase and carveol synthase.

18. The method of claim 17, wherein said second plant cell is transformed with a nucleotide sequence encoding a carveol synthase protein, and said third plant cell is transformed with a nucleotide sequence encoding GPP synthase.

19. A method of producing GPP synthase and S-linalool synthase in a plant cell, said method comprising

transforming a first plant cell with a nucleotide sequence encoding a GPP synthase protein operably linked to a promoter that drives expression in a plant cell,

transforming a second plant cell with a nucleotide sequence encoding a S-linalool synthase protein operably linked to a promoter that drives expression in a plant cell,

producing a first fertile transgenic plant from said first plant cell,

producing a second fertile transgenic plant from said second plant cell,

crossing said first and second fertile transgenic plants; and

recovering progeny from said cross which express GPP synthase and S-linalool synthase.

20. A transformed plant cell having a manipulated metabolic pathway by having stably incorporated into its genome at least one nucleotide sequence operably linked to a promoter that drives expression in a plant cell,

wherein said nucleotide sequence is selected from the group consisting of:

a) a nucleotide sequence encoding a limonene synthase protein,

b) a nucleotide sequence encoding a GPP synthase protein,

c) a nucleotide sequence encoding a carveol synthase protein,

d) a nucleotide sequence encoding a S-linalool synthase protein,

e) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,

f) a nucleotide sequence encoding a GPP synthase protein having the amino acid sequence set forth in SEQ ID NO:4,

g) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,

h) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,

i) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,

j) a nucleotide sequence encoding a GPP synthase protein as as set forth in SEQ ID NO:3,

k) a nucleotide sequence encoding a carveol synthase protein as forth in SEQ ID NO:5,

l) a nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,

m) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h, i, k or l.

21. A transformed plant having a manipulated metabolic pathway by having stably incorporated into its genome at least one nucleotide sequence operably linked to a promoter that drives expression in a plant cell,

wherein said nucleotide sequence is selected from the group consisting of:

a) a nucleotide sequence encoding a limonene synthase protein,

b) a nucleotide sequence encoding a GPP synthase protein,

c) a nucleotide sequence encoding a carveol synthase protein,

d) a nucleotide sequence encoding a S-linalool synthase protein,

e) a nucleotide sequence encoding a limonene synthase protein having the amino acid sequence set forth in SEQ ID NO:2,

f) a nucleotide sequence encoding a GPP synthase protein having the amino acid sequence set forth in SEQ ID NO:4,

g) a nucleotide sequence encoding a carveol synthase protein having the amino acid sequence set forth in SEQ ID NO:6,

h) a nucleotide sequence encoding a S-linalool synthase protein having the amino acid sequence set forth in SEQ ID NO:8,

i) a nucleotide sequence encoding a limonene synthase protein as set forth in SEQ ID NO:1,

j) a nucleotide sequence encoding a GPP synthase protein as as set forth in SEQ ID NO:3,

k) a nucleotide sequence encoding a carveol synthase protein as forth in SEQ ID NO:5,

l) a nucleotide sequence encoding a S-linalool synthase protein as set forth in SEQ ID NO:7,

m) a nucleotide sequence corresponding to a portion of an antisense sequence for said nucleotide sequences of a, b, c, d, e, f, g, h, i, j, k or l.

22. The plant of claim 21 wherein said plant is monocotyledonous.

23. The plant of claim 22 wherein said monocot plant is a corn, wheat, rice, oat, rye or sorghum plant.

24. The plant of claim 21 wherein said promoter is a constitutive promoter.

25. The plant of claim 24 wherein said constitutive promoter is a ubiquitin promoter.

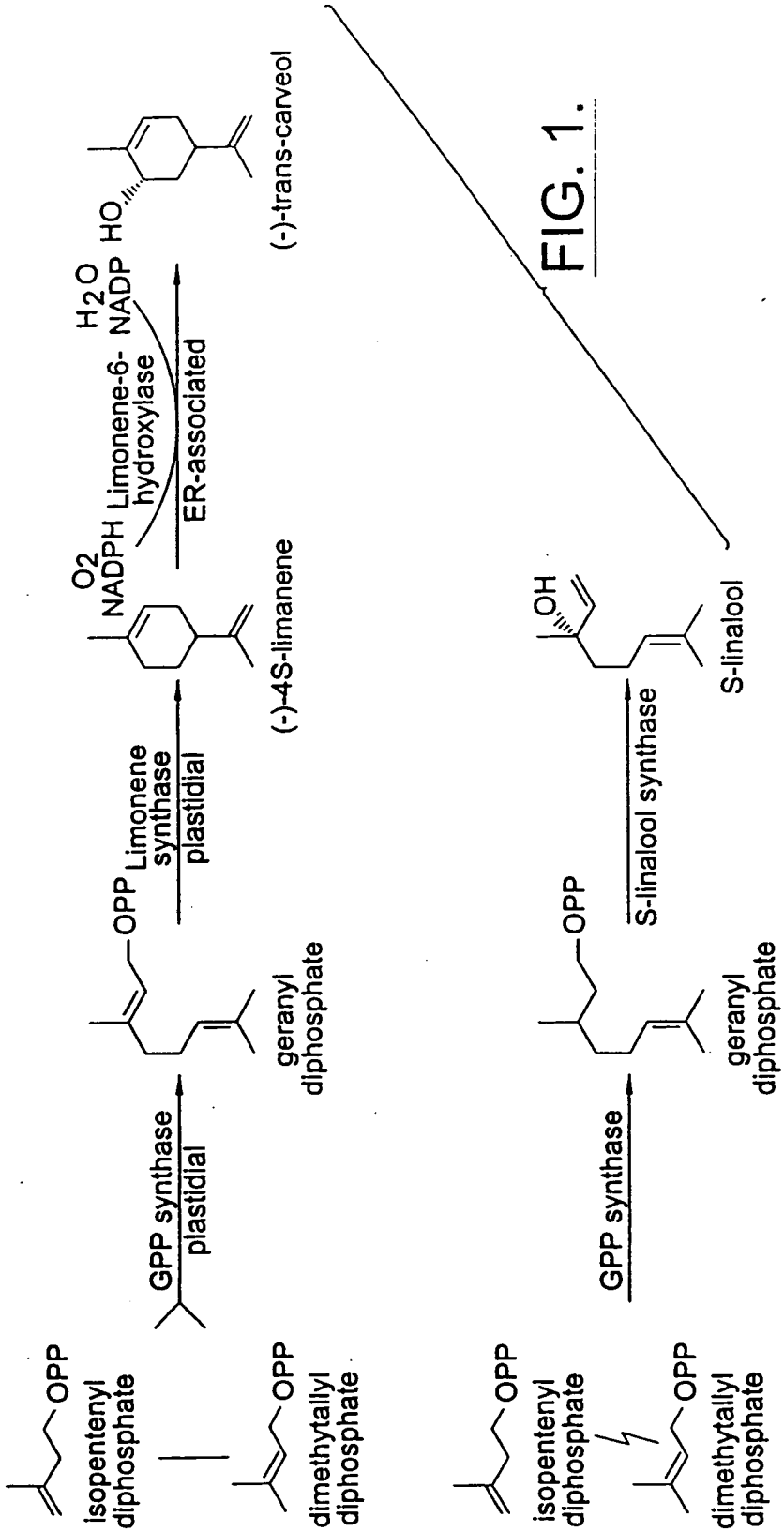
26. The plant of claim 21 wherein said promoter is a tissue-specific promoter.

27. The plant of claim 26 wherein said tissue-specific promoter is a root-specific promoter.

28. The plant of any of claims 24-27 wherein said plant is a dicot.

29. Seed of the plant of any of claims 21-27.

30. Seed of the plant of claim 28.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OPP
dimethytallyl diphosphate
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O2 NADPH
Limonene-6-hydroxylase
ER-associated
H2O NADP
HO
(-)-trans-carveol
OPP
isopentenyl diphosphate
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S-linalool synthase
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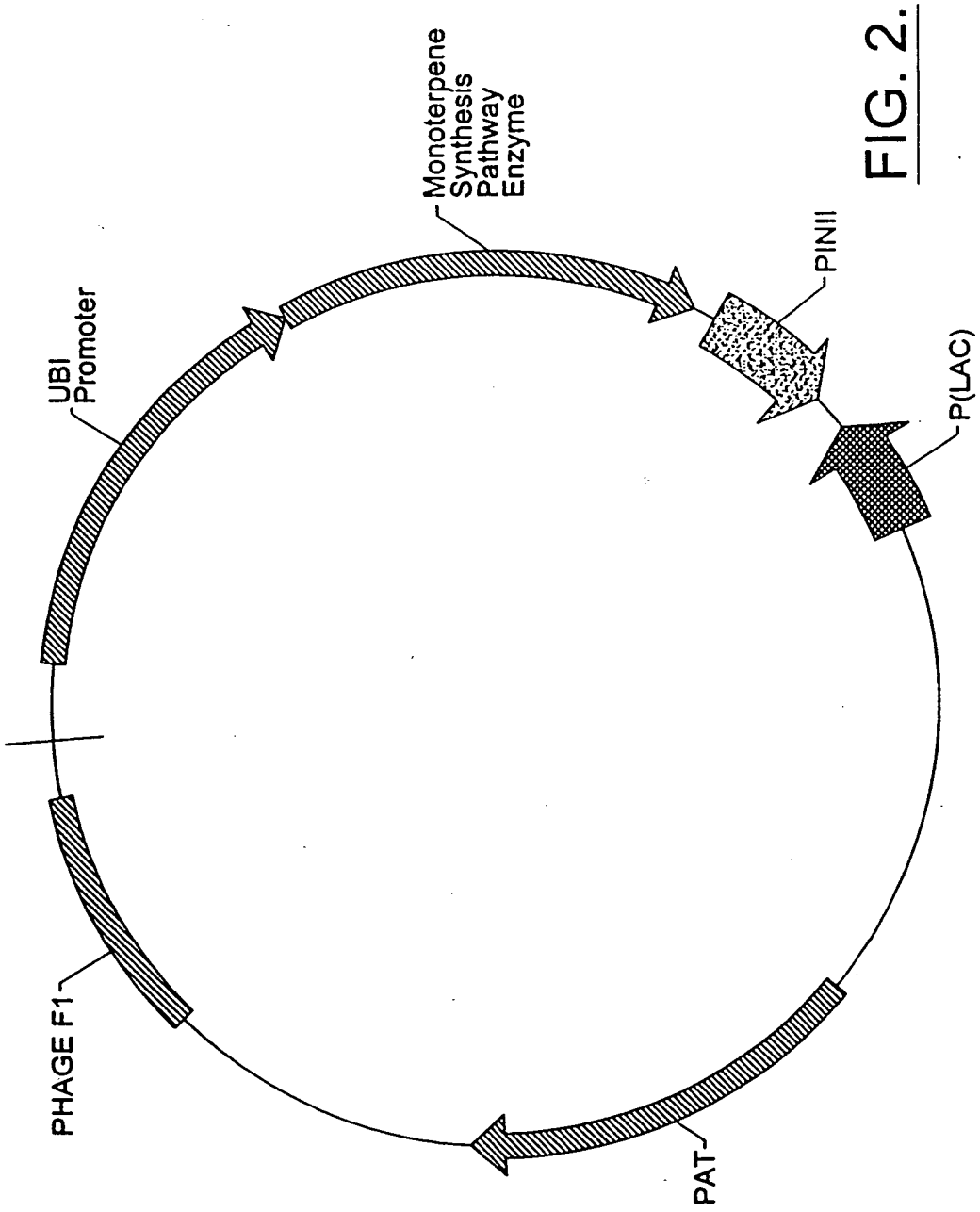
FIG. 1.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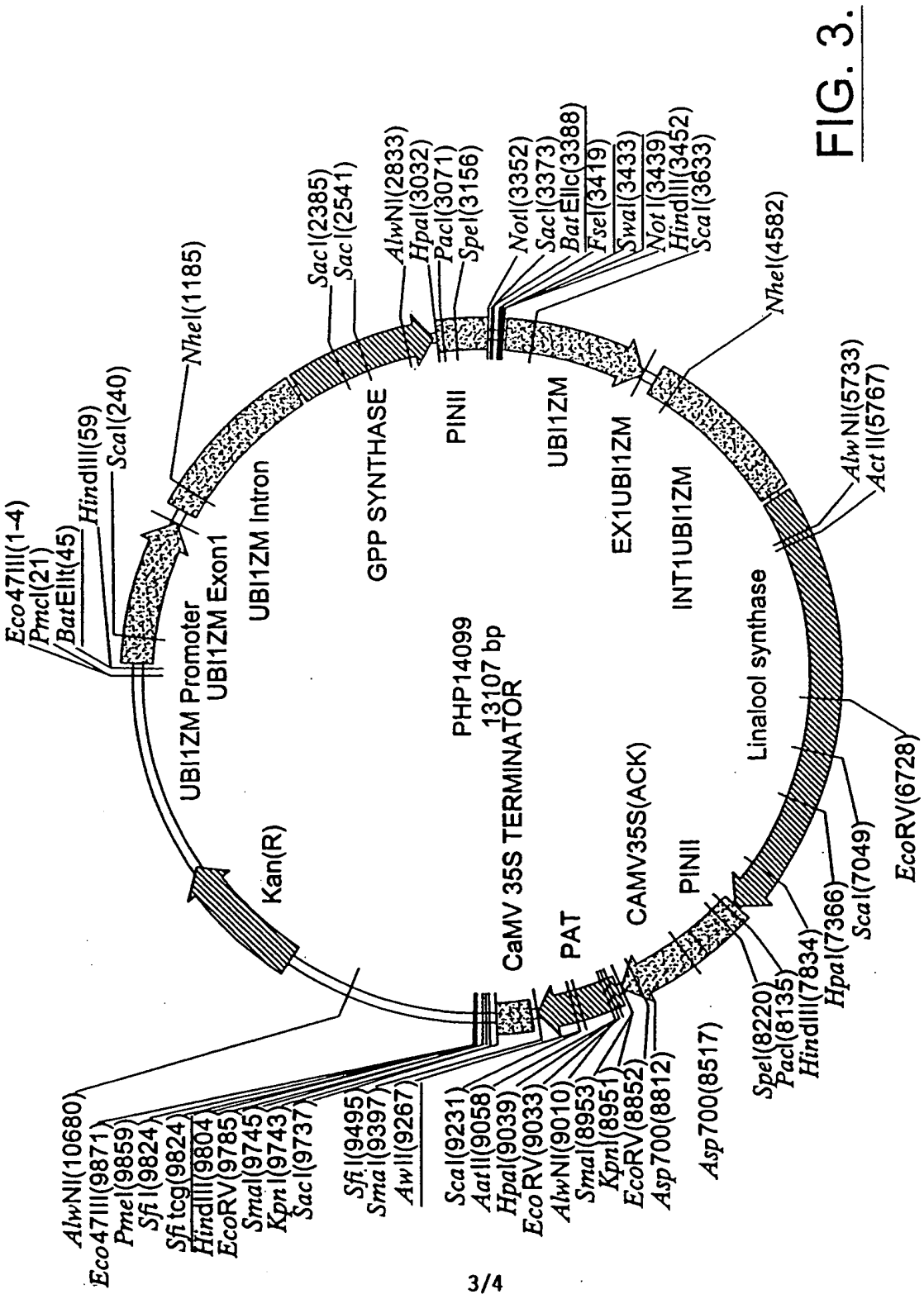
Promoter

FIG. 2.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UBI1ZM Promoter
UBI1ZM Exon1
UBI1ZM Intron
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INT1UBI1ZM
Linalool synthase
PINII
CAMV35S(ACK)
PAT
CaMV 35S TERMINATOR
Kan(R)
Eco47III(1-4)
PmeI(21)
BatEIIt(45)
HindIII(59)
ScaI(240)
NheI(1185)
SacI(2385)
SacI(2541)
AlwNI(2833)
HpaI(3032)
PacI(3071)
SpeI(3156)
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SacI(3373)
BatEIIc(3388)
FseI(3419)
SwaI(3433)
NotI(3439)
HindIII(3452)
ScaI(3633)
NheI(4582)
AlwNI(5733)
ActII(5767)
EcoRV(6728)
ScaI(7049)
HpaI(7366)
HindIII(7834)
PacI(8135)
SpeI(8220)
Asp700(8517)
EcoRV(8812)
EcoRV(8852)
KpnI(8951)
SmaI(8953)
AlwNI(9010)
EcoRV(9033)
HpaI(9039)
AatII(9058)
ScaI(9231)
AwII(9267)
SmaI(9397)
SfiI(9495)
SacI(9737)
KpnI(9743)
SmaI(9745)
EcoRV(9785)
HindIII(9804)
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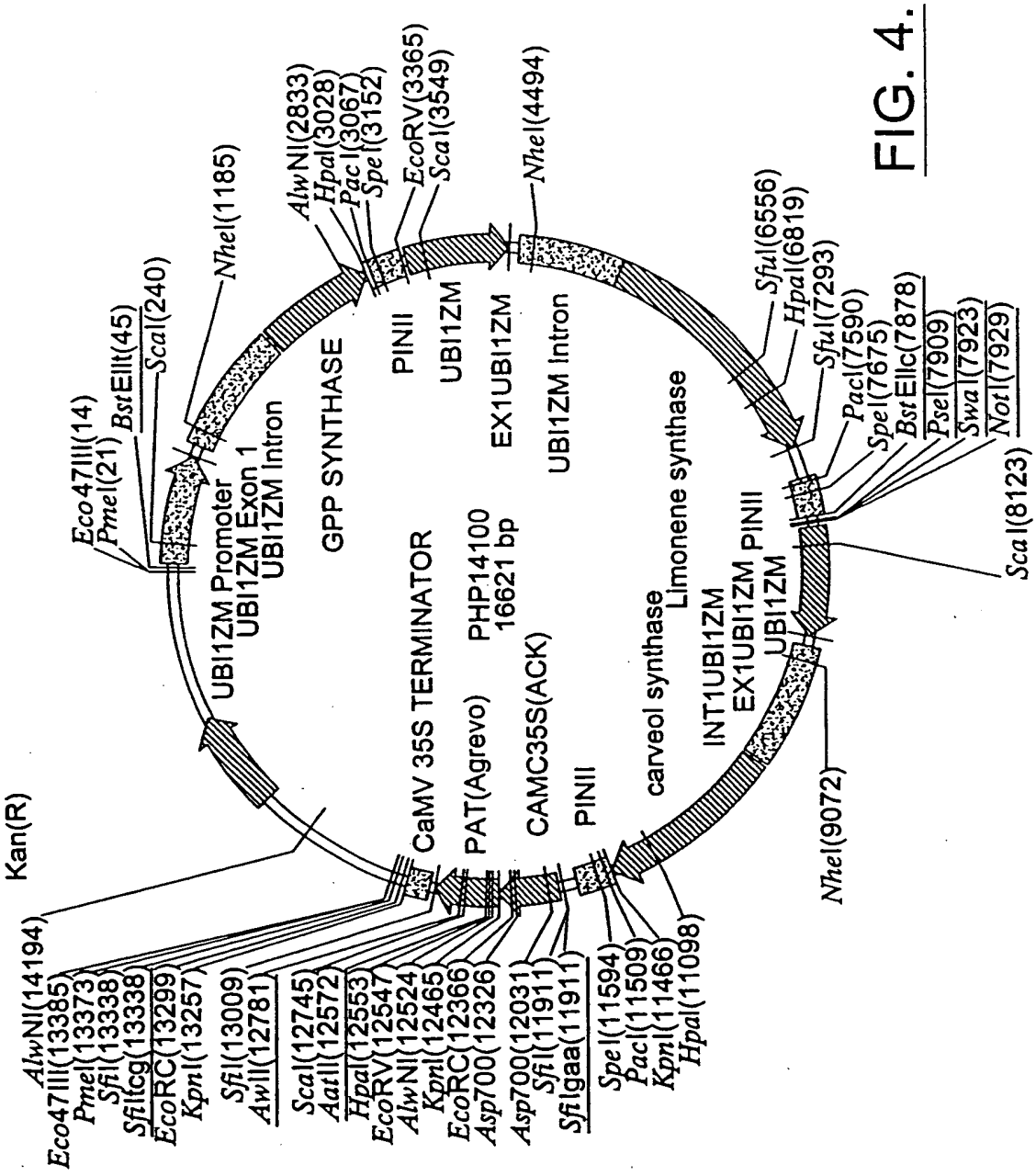
PmeI(9859)
Eco47III(9871)
AlwNI(10680)

FIG. 3.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CaMV 35S TERMINATOR
PAT(Agrevo)
CAMC35S(ACK)
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carveol synthase
Limonene synthase
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EX1UBI1ZM PINII
UBI1ZM
UBI1ZM Intron
EX1UBI1ZM
UBI1ZM
PINII
GPP SYNTHASE
UBI1ZM Promoter
UBI1ZM Exon 1
UBI1ZM Intron
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AlwNI(14194)
Eco47III(13385)
PmeI(13373)
SfiI(13338)
SfiIcg(13338)
EcoRC(13299)
KpnI(13257)
SfiI(13009)
AwII(12781)
ScaI(12745)
AatII(12572)
HpaI(12553)
EcoRV(12547)
AlwNI(12524)
KpnI(12465)
EcoRC(12366)
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SfiI(11911)
SfiIgaa(11911)
SpeI(11594)
PacI(11509)
KpnI(11466)
HpaI(11098)
NheI(9072)
ScaI(8123)
NotI(7929)
SwaI(7923)
PseI(7909)
BstEIIc(7878)
SpeI(7675)
PacI(7590)
SfuI(7293)
HpaI(6819)
SfuI(6556)
EcoRV(3365)
ScaI(3549)
NheI(4494)
SpeI(3152)
PacI(3067)
HpaI(3028)
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ScaI(240)
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FIG. 4.

SEQUENCE LISTING

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Yalpani, Nasser

<120> Limonene and Other Downstream Metabolites of Geranyl Pyrophosphate for Insect Control in Plants

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<141> 1998-10-14

<150> 08/449,061
<151> 1995-05-24

<150> 08/153,544
<151> 1993-11-16

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                               Met Ala Leu Lys Val Leu Ser Val
                                1               5

gca act caa atg gcg att cct agc aac cta acg aca tgt ctt caa ccc      100
Ala Thr Gln Met Ala Ile Pro Ser Asn Leu Thr Thr Cys Leu Gln Pro
     10                  15                  20

tca cac ttc aaa tct tct cca aaa ctg tta tct agc act aac agt agt      148
Ser His Phe Lys Ser Ser Pro Lys Leu Leu Ser Ser Thr Asn Ser Ser
 25                  30                  35                  40

agt cgg tct cgc ctc cgt gtg tat tgc tcc tcc tcg caa ctc act act      196
Ser Arg Ser Arg Leu Arg Val Tyr Cys Ser Ser Ser Gln Leu Thr Thr
                 45                  50                  55
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gaa aga cga tcc gga aac tac aac cct tct cgt tgg gat gtc aac ttc      244
Glu Arg Arg Ser Gly Asn Tyr Asn Pro Ser Arg Trp Asp Val Asn Phe
            60                  65                  70

atc caa tcg ctt ctc agt gac tat aag gag gac aaa cac gtg att agg      292
Ile Gln Ser Leu Leu Ser Asp Tyr Lys Glu Asp Lys His Val Ile Arg
        75                  80                  85

gct tct gag ctg gtc act ttg gtg aag atg gaa ctg gag aaa gaa acg      340
Ala Ser Glu Leu Val Thr Leu Val Lys Met Glu Leu Glu Lys Glu Thr
    90                  95                  100

gat caa att cga caa ctt gag ttg atc gat gac ttg cag agg atg ggg      388
Asp Gln Ile Arg Gln Leu Glu Leu Ile Asp Asp Leu Gln Arg Met Gly
105                 110                 115                 120

ctg tcc gat cat ttc caa aat gag ttc aaa gaa atc ttg tcc tct ata      436
Leu Ser Asp His Phe Gln Asn Glu Phe Lys Glu Ile Leu Ser Ser Ile
                125                 130                 135

tat ctc gac cat cac tat tac aag aac cct ttt cca aaa gaa gaa agg      484
Tyr Leu Asp His His Tyr Tyr Lys Asn Pro Phe Pro Lys Glu Glu Arg
            140                 145                 150

gat ctc tac tcc aca tct ctt gca ttt agg ctc ctc aga gaa cat ggt      532
Asp Leu Tyr Ser Thr Ser Leu Ala Phe Arg Leu Leu Arg Glu His Gly
        155                 160                 165

ttt caa gtc gca caa gag gta ttc gat agt ttc aag aac gag gag ggt      580
Phe Gln Val Ala Gln Glu Val Phe Asp Ser Phe Lys Asn Glu Glu Gly
    170                 175                 180

gag ttc aaa gaa agc ctt agc gac gac acc aga gga ttg ttg caa ctg      628
Glu Phe Lys Glu Ser Leu Ser Asp Asp Thr Arg Gly Leu Leu Gln Leu
185                 190                 195                 200

tat gaa gct tcc ttt ctg ttg acg gaa ggc gaa acc acg ctc gag tca      676
Tyr Glu Ala Ser Phe Leu Leu Thr Glu Gly Glu Thr Thr Leu Glu Ser
                205                 210                 215

gcg agg gaa ttc gcc acc aaa ttt ttg gag gaa aaa gtg aac gag ggt      724
Ala Arg Glu Phe Ala Thr Lys Phe Leu Glu Glu Lys Val Asn Glu Gly
            220                 225                 230

ggt gtt gat ggc gac ctt tta aca aga atc gca tat tct ttg gac atc      772
Gly Val Asp Gly Asp Leu Leu Thr Arg Ile Ala Tyr Ser Leu Asp Ile
        235                 240                 245

cct ctt cat tgg agg att aaa agg cca aat gca cct gtg tgg atc gaa      820
Pro Leu His Trp Arg Ile Lys Arg Pro Asn Ala Pro Val Trp Ile Glu
    250                 255                 260

tgg tat agg aag agg ccc gac atg aat cca gta gtg ttg gag ctt gcc      868
Trp Tyr Arg Lys Arg Pro Asp Met Asn Pro Val Val Leu Glu Leu Ala
265                 270                 275                 280
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ata ctc gac tta aat att gtt caa gca caa ttt caa gaa gag ctc aaa      916
Ile Leu Asp Leu Asn Ile Val Gln Ala Gln Phe Gln Glu Glu Leu Lys
                285                 290                 295

gaa tcc ttc agg tgg tgg aga aat act ggg ttt gtt gag aag ctg ccc      964
Glu Ser Phe Arg Trp Trp Arg Asn Thr Gly Phe Val Glu Lys Leu Pro
            300                 305                 310

ttc gca agg gat aga ctg gtg gaa tgc tac ttt tgg aat act ggg atc     1012
Phe Ala Arg Asp Arg Leu Val Glu Cys Tyr Phe Trp Asn Thr Gly Ile
        315                 320                 325

atc gag cca cgt cag cat gca agt gca agg ata atg atg ggc aaa gtc     1060
Ile Glu Pro Arg Gln His Ala Ser Ala Arg Ile Met Met Gly Lys Val
    330                 335                 340

aac gct ctg att acg gtg atc gat gat att tat gat gtc tat ggc acc     1108
Asn Ala Leu Ile Thr Val Ile Asp Asp Ile Tyr Asp Val Tyr Gly Thr
345                 350                 355                 360

tta gaa gaa ctc gaa caa ttc act gac ctc att cga aga tgg gat ata     1156
Leu Glu Glu Leu Glu Gln Phe Thr Asp Leu Ile Arg Arg Trp Asp Ile
                365                 370                 375

aac tca atc gac caa ctt ccc gat tac atg caa ctg tgc ttt ctt gca     1204
Asn Ser Ile Asp Gln Leu Pro Asp Tyr Met Gln Leu Cys Phe Leu Ala
            380                 385                 390

ctc aac aac ttc gtc gat gat aca tcg tac gat gtt atg aag gag aaa     1252
Leu Asn Asn Phe Val Asp Asp Thr Ser Tyr Asp Val Met Lys Glu Lys
        395                 400                 405

ggc gtc aac gtt ata ccc tac ctg cgg caa tcg tgg gtt gat ttg gcg     1300
Gly Val Asn Val Ile Pro Tyr Leu Arg Gln Ser Trp Val Asp Leu Ala
    410                 415                 420

gat aag tat atg gta gag gca cgg tgg ttc tac ggc ggg cac aaa cca     1348
Asp Lys Tyr Met Val Glu Ala Arg Trp Phe Tyr Gly Gly His Lys Pro
425                 430                 435                 440

agt ttg gaa gag tat ttg gag aac tca tgg cag tcg ata agt ggg ccc     1396
Ser Leu Glu Glu Tyr Leu Glu Asn Ser Trp Gln Ser Ile Ser Gly Pro
                445                 450                 455

tgt atg tta acg cac ata ttc ttc cga gta aca gat tcg ttc aca aag     1444
Cys Met Leu Thr His Ile Phe Phe Arg Val Thr Asp Ser Phe Thr Lys
            460                 465                 470

gag acc gtc gac agt ttg tac aaa tac cac gat tta gtt cgt tgg tca     1492
Glu Thr Val Asp Ser Leu Tyr Lys Tyr His Asp Leu Val Arg Trp Ser
        475                 480                 485

tcc ttc gtt ctg cgg ctt gct gat gat ttg gga acc tcg gtg gaa gag     1540
Ser Phe Val Leu Arg Leu Ala Asp Asp Leu Gly Thr Ser Val Glu Glu
    490                 495                 500

gtg agc aga ggg gat gtg ccg aaa tca ctt cag tgc tac atg agt gac     1588
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Val Ser Arg Gly Asp Val Pro Lys Ser Leu Gln Cys Tyr Met Ser Asp
505                 510                 515                 520

tac aat gca tcg gag gcg gag gcg cgg aag cac gtg aaa tgg ctg ata     1636
Tyr Asn Ala Ser Glu Ala Glu Ala Arg Lys His Val Lys Trp Leu Ile
                525                 530                 535

gcg gag gtg tgg aag aag atg aat gcg gag agg gtg tcg aag gat tct     1684
Ala Glu Val Trp Lys Lys Met Asn Ala Glu Arg Val Ser Lys Asp Ser
            540                 545                 550

cca ttc ggc aaa gat ttt ata gga tgt gca gtt gat tta gga agg atg     1732
Pro Phe Gly Lys Asp Phe Ile Gly Cys Ala Val Asp Leu Gly Arg Met
        555                 560                 565

gcg cag ttg atg tac cat aat gga gat ggg cac ggc aca caa cac cct     1780
Ala Gln Leu Met Tyr His Asn Gly Asp Gly His Gly Thr Gln His Pro
    570                 575                 580

att ata cat caa caa atg acc aga acc tta ttc gag ccc ttt gca         1825
Ile Ile His Gln Gln Met Thr Arg Thr Leu Phe Glu Pro Phe Ala
585                 590                 595

tgagagatga tgacgagcca tcgtttactt acttaaattc taccaaagtt tttcgaaggc   1885
atagttcgta atttttcaag caccaataaa taaggagaat cggctcaaac aaacgtggca   1945
tttgccacca cgtgagcaca agggagagtc tgtcgtcgtt tatggatgaa ctattcaatt   2005
tttatgcatg taataattaa gttcaagttc aagagccttc tgcatattta actatgtatt   2065
tgaatttatc gagtgtgatt ttctgtcttt ggcaacatat atttttgtca tatgtggcat   2125
cttattatga tatcatacag tgtttatgga tgatatgata ctatc                  2170
```

<210> 2
<211> 599
<212> PRT
<213> Mentha spicata

<400> 2

```
Met Ala Leu Lys Val Leu Ser Val Ala Thr Gln Met Ala Ile Pro Ser
 1               5                  10                  15
Asn Leu Thr Thr Cys Leu Gln Pro Ser His Phe Lys Ser Ser Pro Lys
            20                  25                  30
Leu Leu Ser Ser Thr Asn Ser Ser Ser Arg Ser Arg Leu Arg Val Tyr
        35                  40                  45
Cys Ser Ser Ser Gln Leu Thr Thr Glu Arg Arg Ser Gly Asn Tyr Asn
    50                  55                  60
Pro Ser Arg Trp Asp Val Asn Phe Ile Gln Ser Leu Leu Ser Asp Tyr
65                  70                  75                  80
Lys Glu Asp Lys His Val Ile Arg Ala Ser Glu Leu Val Thr Leu Val
                85                  90                  95
Lys Met Glu Leu Glu Lys Glu Thr Asp Gln Ile Arg Gln Leu Glu Leu
            100                 105                 110
Ile Asp Asp Leu Gln Arg Met Gly Leu Ser Asp His Phe Gln Asn Glu
        115                 120                 125
Phe Lys Glu Ile Leu Ser Ser Ile Tyr Leu Asp His His Tyr Tyr Lys
    130                 135                 140
Asn Pro Phe Pro Lys Glu Glu Arg Asp Leu Tyr Ser Thr Ser Leu Ala
145                 150                 155                 160
Phe Arg Leu Leu Arg Glu His Gly Phe Gln Val Ala Gln Glu Val Phe
```

```
                165                 170                 175
Asp Ser Phe Lys Asn Glu Glu Gly Glu Phe Lys Glu Ser Leu Ser Asp
            180                 185                 190
Asp Thr Arg Gly Leu Leu Gln Leu Tyr Glu Ala Ser Phe Leu Leu Thr
        195                 200                 205
Glu Gly Glu Thr Thr Leu Glu Ser Ala Arg Glu Phe Ala Thr Lys Phe
    210                 215                 220
Leu Glu Glu Lys Val Asn Glu Gly Gly Val Asp Gly Asp Leu Leu Thr
225                 230                 235                 240
Arg Ile Ala Tyr Ser Leu Asp Ile Pro Leu His Trp Arg Ile Lys Arg
                245                 250                 255
Pro Asn Ala Pro Val Trp Ile Glu Trp Tyr Arg Lys Arg Pro Asp Met
            260                 265                 270
Asn Pro Val Val Leu Glu Leu Ala Ile Leu Asp Leu Asn Ile Val Gln
        275                 280                 285
Ala Gln Phe Gln Glu Glu Leu Lys Glu Ser Phe Arg Trp Trp Arg Asn
    290                 295                 300
Thr Gly Phe Val Glu Lys Leu Pro Phe Ala Arg Asp Arg Leu Val Glu
305                 310                 315                 320
Cys Tyr Phe Trp Asn Thr Gly Ile Ile Glu Pro Arg Gln His Ala Ser
                325                 330                 335
Ala Arg Ile Met Met Gly Lys Val Asn Ala Leu Ile Thr Val Ile Asp
            340                 345                 350
Asp Ile Tyr Asp Val Tyr Gly Thr Leu Glu Glu Leu Glu Gln Phe Thr
        355                 360                 365
Asp Leu Ile Arg Arg Trp Asp Ile Asn Ser Ile Asp Gln Leu Pro Asp
    370                 375                 380
Tyr Met Gln Leu Cys Phe Leu Ala Leu Asn Asn Phe Val Asp Asp Thr
385                 390                 395                 400
Ser Tyr Asp Val Met Lys Glu Lys Gly Val Asn Val Ile Pro Tyr Leu
                405                 410                 415
Arg Gln Ser Trp Val Asp Leu Ala Asp Lys Tyr Met Val Glu Ala Arg
            420                 425                 430
Trp Phe Tyr Gly Gly His Lys Pro Ser Leu Glu Glu Tyr Leu Glu Asn
        435                 440                 445
Ser Trp Gln Ser Ile Ser Gly Pro Cys Met Leu Thr His Ile Phe Phe
    450                 455                 460
Arg Val Thr Asp Ser Phe Thr Lys Glu Thr Val Asp Ser Leu Tyr Lys
465                 470                 475                 480
Tyr His Asp Leu Val Arg Trp Ser Ser Phe Val Leu Arg Leu Ala Asp
                485                 490                 495
Asp Leu Gly Thr Ser Val Glu Glu Val Ser Arg Gly Asp Val Pro Lys
            500                 505                 510
Ser Leu Gln Cys Tyr Met Ser Asp Tyr Asn Ala Ser Glu Ala Glu Ala
        515                 520                 525
Arg Lys His Val Lys Trp Leu Ile Ala Glu Val Trp Lys Lys Met Asn
    530                 535                 540
Ala Glu Arg Val Ser Lys Asp Ser Pro Phe Gly Lys Asp Phe Ile Gly
545                 550                 555                 560
Cys Ala Val Asp Leu Gly Arg Met Ala Gln Leu Met Tyr His Asn Gly
                565                 570                 575
Asp Gly His Gly Thr Gln His Pro Ile Ile His Gln Gln Met Thr Arg
            580                 585                 590
Thr Leu Phe Glu Pro Phe Ala
        595
```

<210> 3

<211> 1131
<212> DNA
<213> Mentha spicata

<220>
<221> misc_feature
<222> (0)...(0)
<223> GPP Synthase

<221> CDS
<222> (6)...(944)

<400> 3

```
tcaaa atg gcc att aat ctc tcc cat atc aac tcc aaa aca tgt ttc cct      50
      Met Ala Ile Asn Leu Ser His Ile Asn Ser Lys Thr Cys Phe Pro
       1               5                  10                  15

ctc aaa aca aga tct gat ctc agc cgt tct tct tcc gcg cgt tgc atg      98
Leu Lys Thr Arg Ser Asp Leu Ser Arg Ser Ser Ser Ala Arg Cys Met
                20                  25                  30

cca act gcc gcc gct gcc gcc ttc ccc act atc gcc acc gcc gcc caa     146
Pro Thr Ala Ala Ala Ala Ala Phe Pro Thr Ile Ala Thr Ala Ala Gln
            35                  40                  45

agt cag ccg tac tgg gcc gcc atc gag gcc gac ata gag aga tac ctg     194
Ser Gln Pro Tyr Trp Ala Ala Ile Glu Ala Asp Ile Glu Arg Tyr Leu
        50                  55                  60

aag aaa tcc atc aca ata agg ccg ccg gag aca gtt ttc ggg ccc atg     242
Lys Lys Ser Ile Thr Ile Arg Pro Pro Glu Thr Val Phe Gly Pro Met
    65                  70                  75

cac cac ctc acc ttc gcc gcc cca gcc acc gcc gcc tcc acc cta tgc     290
His His Leu Thr Phe Ala Ala Pro Ala Thr Ala Ala Ser Thr Leu Cys
80                  85                  90                  95

ttg gcg gcg tgc gag ctc gtc ggc ggc gac cga agc caa gcc atg gca     338
Leu Ala Ala Cys Glu Leu Val Gly Gly Asp Arg Ser Gln Ala Met Ala
                100                 105                 110

gcc gcg gcg gcg atc cat ctc gtg cac gcg gca gcc tac gtc cac gag     386
Ala Ala Ala Ala Ile His Leu Val His Ala Ala Ala Tyr Val His Glu
            115                 120                 125

cac ctc cct cta acc gac ggg tcg agg ccc gta tcc aag ccc gca atc     434
His Leu Pro Leu Thr Asp Gly Ser Arg Pro Val Ser Lys Pro Ala Ile
        130                 135                 140

cag cac aag tac ggc ccg aac gtc gag ctc ctc acc gga gac ggg att     482
Gln His Lys Tyr Gly Pro Asn Val Glu Leu Leu Thr Gly Asp Gly Ile
    145                 150                 155

gtc ccg ttc ggg ttt gag ttg ctg gcc ggg tca gtg gac ccg gcc cga     530
Val Pro Phe Gly Phe Glu Leu Leu Ala Gly Ser Val Asp Pro Ala Arg
160                 165                 170                 175
```

```
aca gac gac ccg gat agg att ctg aga gtt ata ata gag atc agt cgg      578
Thr Asp Asp Pro Asp Arg Ile Leu Arg Val Ile Ile Glu Ile Ser Arg
                180                 185                 190

gcc ggc ggg ccg gag gga atg ata agc ggg ctg cat agg gaa gaa gaa      626
Ala Gly Gly Pro Glu Gly Met Ile Ser Gly Leu His Arg Glu Glu Glu
            195                 200                 205

att gtt gat gga aat acg agt tta gac ttc att gaa tat gtg tgc aag      674
Ile Val Asp Gly Asn Thr Ser Leu Asp Phe Ile Glu Tyr Val Cys Lys
        210                 215                 220

aaa aaa tac ggc gag atg cat gct tgc ggc gcg gct tgt gga gcc ata      722
Lys Lys Tyr Gly Glu Met His Ala Cys Gly Ala Ala Cys Gly Ala Ile
    225                 230                 235

ttg ggc ggc gca gcc gag gag gag att cag aag ctg agg aat ttc ggg      770
Leu Gly Gly Ala Ala Glu Glu Glu Ile Gln Lys Leu Arg Asn Phe Gly
240                 245                 250                 255

ctt tat caa gga act ctc aga gga atg atg gaa atg aaa aat tct cat      818
Leu Tyr Gln Gly Thr Leu Arg Gly Met Met Glu Met Lys Asn Ser His
                260                 265                 270

caa tta att gat gag aat ata att gga aaa ttg aaa gaa ttg gct ctc      866
Gln Leu Ile Asp Glu Asn Ile Ile Gly Lys Leu Lys Glu Leu Ala Leu
            275                 280                 285

gag gag ttg gga ggc ttc cac ggg aag aac gct gag ctg atg tcg agc      914
Glu Glu Leu Gly Gly Phe His Gly Lys Asn Ala Glu Leu Met Ser Ser
        290                 295                 300

ctt gta gcc gag ccg agc ctt tac gcg gct tagagctatt cggatccttc       964
Leu Val Ala Glu Pro Ser Leu Tyr Ala Ala
    305                 310

attgcatttt catgcgacat cttcatattc atattgcata atatttttta agccagttat   1024
ttttttatta tgaatttttt taactgttat tgatttcgaa aatactgaca atcatctaaa   1084
ataaagtaaa tatagtaagg atgaaaaaaa aaaaaaaaaa aaaaaaa                 1131
```

<210> 4
<211> 313
<212> PRT
<213> Mentha spicata

<400> 4

```
Met Ala Ile Asn Leu Ser His Ile Asn Ser Lys Thr Cys Phe Pro Leu
 1               5                  10                  15
Lys Thr Arg Ser Asp Leu Ser Arg Ser Ser Ser Ala Arg Cys Met Pro
            20                  25                  30
Thr Ala Ala Ala Ala Ala Phe Pro Thr Ile Ala Thr Ala Ala Gln Ser
        35                  40                  45
Gln Pro Tyr Trp Ala Ala Ile Glu Ala Asp Ile Glu Arg Tyr Leu Lys
    50                  55                  60
Lys Ser Ile Thr Ile Arg Pro Pro Glu Thr Val Phe Gly Pro Met His
65                  70                  75                  80
His Leu Thr Phe Ala Ala Pro Ala Thr Ala Ala Ser Thr Leu Cys Leu
```

```
                85                  90                  95
Ala Ala Cys Glu Leu Val Gly Gly Asp Arg Ser Gln Ala Met Ala Ala
            100                 105                 110
Ala Ala Ala Ile His Leu Val His Ala Ala Ala Tyr Val His Glu His
        115                 120                 125
Leu Pro Leu Thr Asp Gly Ser Arg Pro Val Ser Lys Pro Ala Ile Gln
    130                 135                 140
His Lys Tyr Gly Pro Asn Val Glu Leu Leu Thr Gly Asp Gly Ile Val
145                 150                 155                 160
Pro Phe Gly Phe Glu Leu Leu Ala Gly Ser Val Asp Pro Ala Arg Thr
                165                 170                 175
Asp Asp Pro Asp Arg Ile Leu Arg Val Ile Ile Glu Ile Ser Arg Ala
            180                 185                 190
Gly Gly Pro Glu Gly Met Ile Ser Gly Leu His Arg Glu Glu Glu Ile
        195                 200                 205
Val Asp Gly Asn Thr Ser Leu Asp Phe Ile Glu Tyr Val Cys Lys Lys
    210                 215                 220
Lys Tyr Gly Glu Met His Ala Cys Gly Ala Ala Cys Gly Ala Ile Leu
225                 230                 235                 240
Gly Gly Ala Ala Glu Glu Glu Ile Gln Lys Leu Arg Asn Phe Gly Leu
                245                 250                 255
Tyr Gln Gly Thr Leu Arg Gly Met Met Glu Met Lys Asn Ser His Gln
            260                 265                 270
Leu Ile Asp Glu Asn Ile Ile Gly Lys Leu Lys Glu Leu Ala Leu Glu
        275                 280                 285
Glu Leu Gly Gly Phe His Gly Lys Asn Ala Glu Leu Met Ser Ser Leu
    290                 295                 300
Val Ala Glu Pro Ser Leu Tyr Ala Ala
305                 310
```

<210> 5
<211> 1762
<212> DNA
<213> Mentha spicata

<220>
<221> misc_feature
<222> (0)...(0)
<223> Carveol Synthase

<221> CDS
<222> (20)...(1507)

<400> 5

```
aaaaaacwaa aaagaaacw atg gag ctc gac ctt ttg tcg gca att ata atc      52
                       Met Glu Leu Asp Leu Leu Ser Ala Ile Ile Ile
                         1               5                  10

ctt gtg gca acc tac atc gta tcc ctc cta atc aac caa tgg cga aaa      100
Leu Val Ala Thr Tyr Ile Val Ser Leu Leu Ile Asn Gln Trp Arg Lys
             15                  20                  25

tcg aaa tcc caa caa aac cta cct ccg agc cct ccg aag ctg ccg gtg      148
Ser Lys Ser Gln Gln Asn Leu Pro Pro Ser Pro Pro Lys Leu Pro Val
         30                  35                  40

atc ggc cac ctc cac ttc ctg tgg gga ggg ctt ccc cag cac gtg ttt      196
```

```
Ile Gly His Leu His Phe Leu Trp Gly Gly Leu Pro Gln His Val Phe
     45                  50                  55

agg agc ata gcc cag aag tac ggg ccg gtg gcg cac gtg cag ctg gga      244
Arg Ser Ile Ala Gln Lys Tyr Gly Pro Val Ala His Val Gln Leu Gly
 60                  65                  70                  75

gaa gtg tac tcg gtg gtg ctg tcg tcg gcg gag gca gcg ccg cag gcg      292
Glu Val Tyr Ser Val Val Leu Ser Ser Ala Glu Ala Ala Pro Gln Ala
                 80                  85                  90

atg aag gtg ctg gac ccg aac ttc gcc gac cgg ttc gac ggc atc ggg      340
Met Lys Val Leu Asp Pro Asn Phe Ala Asp Arg Phe Asp Gly Ile Gly
             95                 100                 105

tcc agg acc atg tgg tac gac aaa gat gac atc atc ttc agc cct tac      388
Ser Arg Thr Met Trp Tyr Asp Lys Asp Asp Ile Ile Phe Ser Pro Tyr
        110                 115                 120

aac gat cac tgg cgc cag atg cgg agg atc tgc gtg aca gag ctg ctg      436
Asn Asp His Trp Arg Gln Met Arg Arg Ile Cys Val Thr Glu Leu Leu
    125                 130                 135

agc ccg aag aac gtc agg tcc ttc ggg tac ata agg cag gag gag atc      484
Ser Pro Lys Asn Val Arg Ser Phe Gly Tyr Ile Arg Gln Glu Glu Ile
140                 145                 150                 155

gag cgc ctc atc cgg ctg ctc ggg tcg tcg ggg gga gcg ccg gtc gac      532
Glu Arg Leu Ile Arg Leu Leu Gly Ser Ser Gly Gly Ala Pro Val Asp
                160                 165                 170

gtg acg gag gag gtg tcg aag atg tcg tgt gtc gtc gtg tgc agg gcg      580
Val Thr Glu Glu Val Ser Lys Met Ser Cys Val Val Val Cys Arg Ala
            175                 180                 185

gcg ttc ggg agt gtg ctc aag gac cag ggt tcg ttg gcg gag ttg gtg      628
Ala Phe Gly Ser Val Leu Lys Asp Gln Gly Ser Leu Ala Glu Leu Val
        190                 195                 200

aag gag tcg ctg gca ttg gcg tcc ggg ttt gag ctg gcg gat ctc tac      676
Lys Glu Ser Leu Ala Leu Ala Ser Gly Phe Glu Leu Ala Asp Leu Tyr
    205                 210                 215

cct tcc tca tgg ctc ctc aac ctg ctt agc ttg aac aag tac agg ttg      724
Pro Ser Ser Trp Leu Leu Asn Leu Leu Ser Leu Asn Lys Tyr Arg Leu
220                 225                 230                 235

cag agg atg cgc cgc cgc ctc gat cac atc ctt gat ggg ttc ctg gag      772
Gln Arg Met Arg Arg Arg Leu Asp His Ile Leu Asp Gly Phe Leu Glu
                240                 245                 250

gag cat agg gag aag aag agc ggc gac ttt gga ggc gag gac atc gtc      820
Glu His Arg Glu Lys Lys Ser Gly Asp Phe Gly Gly Glu Asp Ile Val
            255                 260                 265

gac gtt ctt ttc agg atg cag ccg ggc agc gac agc aaa att ccc att      868
Asp Val Leu Phe Arg Met Gln Pro Gly Ser Asp Ser Lys Ile Pro Ile
```

```
        270                 275                 280

act tcc aat tgc atc aag ggt ttc att ttc gac acc ttc tcc gcg gga      916
Thr Ser Asn Cys Ile Lys Gly Phe Ile Phe Asp Thr Phe Ser Ala Gly
    285                 290                 295

gct gaa acg tct tcg acg acc atc tca tgg gcg ttg tcg gaa ctg atg      964
Ala Glu Thr Ser Ser Thr Thr Ile Ser Trp Ala Leu Ser Glu Leu Met
300                 305                 310                 315

agg aat ccg gcg aag atg gcc aag gtg cag gcg gag gta aga gag gcg     1012
Arg Asn Pro Ala Lys Met Ala Lys Val Gln Ala Glu Val Arg Glu Ala
                320                 325                 330

ctc aag gga aag aca gtc gtg gat ttg agc gag gtg caa gag cta aaa     1060
Leu Lys Gly Lys Thr Val Val Asp Leu Ser Glu Val Gln Glu Leu Lys
            335                 340                 345

tac ctg aga tcg gtg tta aag gag act ctg agg ctg cac cct ccc ttt     1108
Tyr Leu Arg Ser Val Leu Lys Glu Thr Leu Arg Leu His Pro Pro Phe
        350                 355                 360

cca tta atc cca aga caa tcc agg gaa gaa tgc gag gtt aac ggg tac     1156
Pro Leu Ile Pro Arg Gln Ser Arg Glu Glu Cys Glu Val Asn Gly Tyr
    365                 370                 375

acg att ccg gcc aaa act aga atc ttc atc aac gtc tgg gct atc gga     1204
Thr Ile Pro Ala Lys Thr Arg Ile Phe Ile Asn Val Trp Ala Ile Gly
380                 385                 390                 395

agg gat ccc caa tac tgg gaa gat ccc gac acc ttc cgc cct gag aga     1252
Arg Asp Pro Gln Tyr Trp Glu Asp Pro Asp Thr Phe Arg Pro Glu Arg
                400                 405                 410

ttc gat gag gtt tcc agg gat ttc atg gga aac gat ttc gag ttc atc     1300
Phe Asp Glu Val Ser Arg Asp Phe Met Gly Asn Asp Phe Glu Phe Ile
            415                 420                 425

cca ttc ggg gcg ggt cga aga atc tgc ccc ggt tta cat ttc ggg ctg     1348
Pro Phe Gly Ala Gly Arg Arg Ile Cys Pro Gly Leu His Phe Gly Leu
        430                 435                 440

gca aat gtt gag atc cca ttg gcg caa ctg ctc tac cac ttc gac tgg     1396
Ala Asn Val Glu Ile Pro Leu Ala Gln Leu Leu Tyr His Phe Asp Trp
    445                 450                 455

aaa ttg cca caa gga atg act gat gcc gac ttg gca ctg acg gag acc     1444
Lys Leu Pro Gln Gly Met Thr Asp Ala Asp Leu Ala Leu Thr Glu Thr
460                 465                 470                 475

cca ggt ctt tct ggg cca aaa aag aaa aat gtt tgc ttg gtt ccc aca     1492
Pro Gly Leu Ser Gly Pro Lys Lys Lys Asn Val Cys Leu Val Pro Thr
                480                 485                 490

ctc tat aaa agt cct taaccactaa gaagttagca taataagaca tctaaaattg     1547
Leu Tyr Lys Ser Pro
            495
```

```
tcataatcat ctaattattg ttacacttct tctatcatgt cattttgaga agtgtcttat   1607
agaggtggcc acggttccgg ttccagttcg gaagcggaac cgaaccatca gttacggttc   1667
tcagcaagaa gcgaaccgtc ccgccccccc tactgtgttt gagatataaa acacataaaa   1727
taaaataaaa aaaacgctat ttttttttaa aaaaa                              1762
```

<210> 6
<211> 496
<212> PRT
<213> Mentha spicata

<400> 6

```
Met Glu Leu Asp Leu Leu Ser Ala Ile Ile Ile Leu Val Ala Thr Tyr
 1               5                  10                  15
Ile Val Ser Leu Leu Ile Asn Gln Trp Arg Lys Ser Lys Ser Gln Gln
            20                  25                  30
Asn Leu Pro Pro Ser Pro Pro Lys Leu Pro Val Ile Gly His Leu His
        35                  40                  45
Phe Leu Trp Gly Gly Leu Pro Gln His Val Phe Arg Ser Ile Ala Gln
    50                  55                  60
Lys Tyr Gly Pro Val Ala His Val Gln Leu Gly Glu Val Tyr Ser Val
65                  70                  75                  80
Val Leu Ser Ser Ala Glu Ala Ala Pro Gln Ala Met Lys Val Leu Asp
                85                  90                  95
Pro Asn Phe Ala Asp Arg Phe Asp Gly Ile Gly Ser Arg Thr Met Trp
            100                 105                 110
Tyr Asp Lys Asp Asp Ile Ile Phe Ser Pro Tyr Asn Asp His Trp Arg
        115                 120                 125
Gln Met Arg Arg Ile Cys Val Thr Glu Leu Leu Ser Pro Lys Asn Val
    130                 135                 140
Arg Ser Phe Gly Tyr Ile Arg Gln Glu Glu Ile Glu Arg Leu Ile Arg
145                 150                 155                 160
Leu Leu Gly Ser Ser Gly Gly Ala Pro Val Asp Val Thr Glu Glu Val
                165                 170                 175
Ser Lys Met Ser Cys Val Val Val Cys Arg Ala Ala Phe Gly Ser Val
            180                 185                 190
Leu Lys Asp Gln Gly Ser Leu Ala Glu Leu Val Lys Glu Ser Leu Ala
        195                 200                 205
Leu Ala Ser Gly Phe Glu Leu Ala Asp Leu Tyr Pro Ser Ser Trp Leu
    210                 215                 220
Leu Asn Leu Leu Ser Leu Asn Lys Tyr Arg Leu Gln Arg Met Arg Arg
225                 230                 235                 240
Arg Leu Asp His Ile Leu Asp Gly Phe Leu Glu Glu His Arg Glu Lys
                245                 250                 255
Lys Ser Gly Asp Phe Gly Gly Glu Asp Ile Val Asp Val Leu Phe Arg
            260                 265                 270
Met Gln Pro Gly Ser Asp Ser Lys Ile Pro Ile Thr Ser Asn Cys Ile
        275                 280                 285
Lys Gly Phe Ile Phe Asp Thr Phe Ser Ala Gly Ala Glu Thr Ser Ser
    290                 295                 300
Thr Thr Ile Ser Trp Ala Leu Ser Glu Leu Met Arg Asn Pro Ala Lys
305                 310                 315                 320
Met Ala Lys Val Gln Ala Glu Val Arg Glu Ala Leu Lys Gly Lys Thr
                325                 330                 335
Val Val Asp Leu Ser Glu Val Gln Glu Leu Lys Tyr Leu Arg Ser Val
            340                 345                 350
Leu Lys Glu Thr Leu Arg Leu His Pro Pro Phe Pro Leu Ile Pro Arg
```

```
      355                 360                 365
Gln Ser Arg Glu Glu Cys Glu Val Asn Gly Tyr Thr Ile Pro Ala Lys
    370                 375                 380
Thr Arg Ile Phe Ile Asn Val Trp Ala Ile Gly Arg Asp Pro Gln Tyr
385                 390                 395                 400
Trp Glu Asp Pro Asp Thr Phe Arg Pro Glu Arg Phe Asp Glu Val Ser
                405                 410                 415
Arg Asp Phe Met Gly Asn Asp Phe Glu Phe Ile Pro Phe Gly Ala Gly
            420                 425                 430
Arg Arg Ile Cys Pro Gly Leu His Phe Gly Leu Ala Asn Val Glu Ile
        435                 440                 445
Pro Leu Ala Gln Leu Leu Tyr His Phe Asp Trp Lys Leu Pro Gln Gly
    450                 455                 460
Met Thr Asp Ala Asp Leu Ala Leu Thr Glu Thr Pro Gly Leu Ser Gly
465                 470                 475                 480
Pro Lys Lys Lys Asn Val Cys Leu Val Pro Thr Leu Tyr Lys Ser Pro
                485                 490                 495
```

<210> 7
<211> 2681
<212> DNA
<213> Clarkia breweri

<220>
<221> misc_feature
<222> (0)...(0)
<223> S-Linalool Synthase

<221> CDS
<222> (28)...(2637)

<400> 7

```
aaccaaacca ccttaaacaa gacaacc atg cag ctc ata aca aat ttc tcc tca      54
                               Met Gln Leu Ile Thr Asn Phe Ser Ser
                                 1               5

tca tca tca gaa ttg cag ttt ctt gtg gat aag gtt aag aga gaa tca      102
Ser Ser Ser Glu Leu Gln Phe Leu Val Asp Lys Val Lys Arg Glu Ser
 10                  15                  20                  25

ttg tct tct tca tca tct aat act cag aat ttg ttt ctc tca act tca      150
Leu Ser Ser Ser Ser Ser Asn Thr Gln Asn Leu Phe Leu Ser Thr Ser
                 30                  35                  40

cct tat gac act gct tgg ctc gcc ctt atc cct cat cct cat cat cac      198
Pro Tyr Asp Thr Ala Trp Leu Ala Leu Ile Pro His Pro His His His
             45                  50                  55

cat cac cat ggc cga ccc atg ttt gaa aaa tgt ctg caa tgg att ctc      246
His His His Gly Arg Pro Met Phe Glu Lys Cys Leu Gln Trp Ile Leu
         60                  65                  70

cat aac cag aca cca caa ggt ttc tgg gca gca gct ggt gac aat att      294
His Asn Gln Thr Pro Gln Gly Phe Trp Ala Ala Ala Gly Asp Asn Ile
     75                  80                  85

tcc gac acc gac gat gac gtc acc ctg gat tgt ctt cta tca acc ttg      342
```

```
Ser Asp Thr Asp Asp Asp Val Thr Leu Asp Cys Leu Leu Ser Thr Leu
 90                  95                 100                 105

gct tgc tta gtt gca ctc aaa agg tgg cag ctt gct ccc gac atg att      390
Ala Cys Leu Val Ala Leu Lys Arg Trp Gln Leu Ala Pro Asp Met Ile
                110                 115                 120

cat aaa gga ttg gaa ttt gta aat aga aac aca gag aga ctt gta atg      438
His Lys Gly Leu Glu Phe Val Asn Arg Asn Thr Glu Arg Leu Val Met
            125                 130                 135

aag cag aag ccg agc gac gtt cct cgt tgg ttc acc atc atg ttc ccg      486
Lys Gln Lys Pro Ser Asp Val Pro Arg Trp Phe Thr Ile Met Phe Pro
        140                 145                 150

gcg atg ctc gag ctt gcc gga gct tcc agt ctc cga gtc gat ttc agc      534
Ala Met Leu Glu Leu Ala Gly Ala Ser Ser Leu Arg Val Asp Phe Ser
    155                 160                 165

gag aat ctt aac aga atc ttg gtg gaa cta tct caa aat agg gat gat      582
Glu Asn Leu Asn Arg Ile Leu Val Glu Leu Ser Gln Asn Arg Asp Asp
170                 175                 180                 185

att ctc aca agg gag gaa gtt gat gag aag aag caa tac tca cca ttg      630
Ile Leu Thr Arg Glu Glu Val Asp Glu Lys Lys Gln Tyr Ser Pro Leu
                190                 195                 200

cta cta ttt cta gaa gca ttg cct gca caa tcc tat gac aat gat gtt      678
Leu Leu Phe Leu Glu Ala Leu Pro Ala Gln Ser Tyr Asp Asn Asp Val
            205                 210                 215

cta aag caa att ata gac aag aac ttg agc aat gat ggt tct tta ttg      726
Leu Lys Gln Ile Ile Asp Lys Asn Leu Ser Asn Asp Gly Ser Leu Leu
        220                 225                 230

caa tcg cct tct gct aca gca aga gca tac atg ata aca gga aat acc      774
Gln Ser Pro Ser Ala Thr Ala Arg Ala Tyr Met Ile Thr Gly Asn Thr
    235                 240                 245

aga tgc tta tcg tat cta cac tct tta aca aat agc tgc tct aat gga      822
Arg Cys Leu Ser Tyr Leu His Ser Leu Thr Asn Ser Cys Ser Asn Gly
250                 255                 260                 265

gga gta cca tca ttc tat cct gtt gac gac gac ctc cat gat ctt gtc      870
Gly Val Pro Ser Phe Tyr Pro Val Asp Asp Asp Leu His Asp Leu Val
                270                 275                 280

atg gtg aat caa ctg aca agg tcg ggt ttg act gaa cat ctc atc ccg      918
Met Val Asn Gln Leu Thr Arg Ser Gly Leu Thr Glu His Leu Ile Pro
            285                 290                 295

gag att gac cac ctt cta ctc aaa gtt caa aag aac tac aaa tac aaa      966
Glu Ile Asp His Leu Leu Leu Lys Val Gln Lys Asn Tyr Lys Tyr Lys
        300                 305                 310

aaa gca tca cca aaa tca ttg tat agc att gct gcg gaa cta tac agg     1014
Lys Ala Ser Pro Lys Ser Leu Tyr Ser Ile Ala Ala Glu Leu Tyr Arg
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315 320 325

gat tca tta gca ttt tgg ttg ctt cga gtc aat aat cac tgg gta tca 1062
Asp Ser Leu Ala Phe Trp Leu Leu Arg Val Asn Asn His Trp Val Ser
330 335 340 345

cca tca att ttt tgt tgg ttt tta gat gac gac gaa atc cgt gat cac 1110
Pro Ser Ile Phe Cys Trp Phe Leu Asp Asp Asp Glu Ile Arg Asp His
350 355 360

atc gaa aca aac tac gag gaa ttt gct gcc gtg ctt ctt aat gtg tat 1158
Ile Glu Thr Asn Tyr Glu Glu Phe Ala Ala Val Leu Leu Asn Val Tyr
365 370 375

cga gct acc gat ctt atg ttc tcc ggc gaa gtc caa ctt gtc gaa gca 1206
Arg Ala Thr Asp Leu Met Phe Ser Gly Glu Val Gln Leu Val Glu Ala
380 385 390

aga tct ttc gct acc aag aat ctt gag aaa ata tta gca aca gga aac 1254
Arg Ser Phe Ala Thr Lys Asn Leu Glu Lys Ile Leu Ala Thr Gly Asn
395 400 405

ata cat aaa act aat gca gat atc tca tct agt ttg cat aag atg atc 1302
Ile His Lys Thr Asn Ala Asp Ile Ser Ser Ser Leu His Lys Met Ile
410 415 420 425

gaa cac gaa cta aga gtt cct tgg acc gca aga atg gac cat gtt gaa 1350
Glu His Glu Leu Arg Val Pro Trp Thr Ala Arg Met Asp His Val Glu
430 435 440

aat cga att tgg atc gaa gaa ata gct tcc agt gct tta tgg ttt gga 1398
Asn Arg Ile Trp Ile Glu Glu Ile Ala Ser Ser Ala Leu Trp Phe Gly
445 450 455

aaa tca tcc tac ctt agg tta tct tgc ttt cac aag atg agt tta cag 1446
Lys Ser Ser Tyr Leu Arg Leu Ser Cys Phe His Lys Met Ser Leu Gln
460 465 470

caa ctc gcg gtg aaa aat tat acg ctt cga caa ttg gtt tac cga gac 1494
Gln Leu Ala Val Lys Asn Tyr Thr Leu Arg Gln Leu Val Tyr Arg Asp
475 480 485

gag ctt gcg gaa gtt gag agg tgg tct aaa gaa aga ggg cta tgt gac 1542
Glu Leu Ala Glu Val Glu Arg Trp Ser Lys Glu Arg Gly Leu Cys Asp
490 495 500 505

atg gga ttt tgt aga gag aaa acc ggg tat tgt tac tac gca ttt gcg 1590
Met Gly Phe Cys Arg Glu Lys Thr Gly Tyr Cys Tyr Tyr Ala Phe Ala
510 515 520

gca agt act tgt ctg ccg tgg agt tcc gac gtg agg ctg gtc ctg acc 1638
Ala Ser Thr Cys Leu Pro Trp Ser Ser Asp Val Arg Leu Val Leu Thr
525 530 535

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Lys Ala Ala Val Val Ile Thr Val Ala Asp Asp Phe Phe Asp Val Glu
540 545 550

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gga tct atg gtt gat ctc gaa aaa tta acg gat gca gtt cgg agg tgg      1734
Gly Ser Met Val Asp Leu Glu Lys Leu Thr Asp Ala Val Arg Arg Trp
    555                 560                 565

gat gcg gaa ggg tta ggc agc cac agc aag aca ata ttt gaa gcc ctg      1782
Asp Ala Glu Gly Leu Gly Ser His Ser Lys Thr Ile Phe Glu Ala Leu
570                 575                 580                 585

gat gat ctt gta aat gaa gtt aga ctc aag tgt ttc caa caa aat gga      1830
Asp Asp Leu Val Asn Glu Val Arg Leu Lys Cys Phe Gln Gln Asn Gly
                590                 595                 600

caa gac atc aaa aac aat ctc caa caa tta tgg tat gaa aca ttc cat      1878
Gln Asp Ile Lys Asn Asn Leu Gln Gln Leu Trp Tyr Glu Thr Phe His
            605                 610                 615

tca tgg ctt atg gaa gct aag tgg gga aag ggg tta aca agt aaa cca      1926
Ser Trp Leu Met Glu Ala Lys Trp Gly Lys Gly Leu Thr Ser Lys Pro
        620                 625                 630

tct gta gat gtg tat ctt gga aat gca atg aca tcc ata gca gct cac      1974
Ser Val Asp Val Tyr Leu Gly Asn Ala Met Thr Ser Ile Ala Ala His
    635                 640                 645

acc atg gtc ctt aca gca tcc tgt ctt cta ggt ccc ggt ttc ccg gtt      2022
Thr Met Val Leu Thr Ala Ser Cys Leu Leu Gly Pro Gly Phe Pro Val
650                 655                 660                 665

cac caa cta tgg tcg caa agg cgc cac cag gac att aca tcc ttg ctc      2070
His Gln Leu Trp Ser Gln Arg Arg His Gln Asp Ile Thr Ser Leu Leu
                670                 675                 680

atg gtc ttg act cgc ttg cta aat gac att caa tcc tac ttg aaa gaa      2118
Met Val Leu Thr Arg Leu Leu Asn Asp Ile Gln Ser Tyr Leu Lys Glu
            685                 690                 695

gaa gac gaa gga aaa ata aac tat gta tgg atg tac atg atc gag aac      2166
Glu Asp Glu Gly Lys Ile Asn Tyr Val Trp Met Tyr Met Ile Glu Asn
        700                 705                 710

aat caa gcg tcg ata gat gac tcg gtt cga cac gtc cag acg ata atc      2214
Asn Gln Ala Ser Ile Asp Asp Ser Val Arg His Val Gln Thr Ile Ile
    715                 720                 725

aat gta aaa aag caa gaa ttc atc caa cgt gtt cta tcg gat caa cat      2262
Asn Val Lys Lys Gln Glu Phe Ile Gln Arg Val Leu Ser Asp Gln His
730                 735                 740                 745

tgc aat ctc cca aag tca ttc aag cag ctc cat ttc tcc tgc ctc aaa      2310
Cys Asn Leu Pro Lys Ser Phe Lys Gln Leu His Phe Ser Cys Leu Lys
                750                 755                 760

gta ttc aac atg ttc ttc aac tcc tcc aac att ttc gac act gat acc      2358
Val Phe Asn Met Phe Phe Asn Ser Ser Asn Ile Phe Asp Thr Asp Thr
            765                 770                 775
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gac ctt ctt ctt gac att cac gaa gct ttt gtt tct cca cca caa gtt      2406
Asp Leu Leu Leu Asp Ile His Glu Ala Phe Val Ser Pro Pro Gln Val
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ccc aaa ttc aaa ccc cac atc aag cca cct cat cag ctt cca gca aca      2454
Pro Lys Phe Lys Pro His Ile Lys Pro Pro His Gln Leu Pro Ala Thr
    795                 800                 805

ctt cag cca cct cat cag ccc caa caa ata atg gtc aat aag aag aag      2502
Leu Gln Pro Pro His Gln Pro Gln Gln Ile Met Val Asn Lys Lys Lys
810                 815                 820                 825

gtg gaa atg gtt tac aaa agc tat cat cat cca ttc aag gtt ttc acc      2550
Val Glu Met Val Tyr Lys Ser Tyr His His Pro Phe Lys Val Phe Thr
                830                 835                 840

ttg cag aag aaa caa agt tcg gga cat ggt aca atg aat cca agg gct      2598
Leu Gln Lys Lys Gln Ser Ser Gly His Gly Thr Met Asn Pro Arg Ala
            845                 850                 855

agt atc tta gca gga ccc aac atc aaa cta tgt ttc agt taacgaatac      2647
Ser Ile Leu Ala Gly Pro Asn Ile Lys Leu Cys Phe Ser
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actaccttgt tattagaaga tgtcaccagt ttcc                              2681
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Leu Val Asp Lys Val Lys Arg Glu Ser Leu Ser Ser Ser Ser Ser Asn
            20                  25                  30
Thr Gln Asn Leu Phe Leu Ser Thr Ser Pro Tyr Asp Thr Ala Trp Leu
        35                  40                  45
Ala Leu Ile Pro His Pro His His His His His His Gly Arg Pro Met
    50                  55                  60
Phe Glu Lys Cys Leu Gln Trp Ile Leu His Asn Gln Thr Pro Gln Gly
65                  70                  75                  80
Phe Trp Ala Ala Ala Gly Asp Asn Ile Ser Asp Thr Asp Asp Asp Val
                85                  90                  95
Thr Leu Asp Cys Leu Leu Ser Thr Leu Ala Cys Leu Val Ala Leu Lys
            100                 105                 110
Arg Trp Gln Leu Ala Pro Asp Met Ile His Lys Gly Leu Glu Phe Val
        115                 120                 125
Asn Arg Asn Thr Glu Arg Leu Val Met Lys Gln Lys Pro Ser Asp Val
    130                 135                 140
Pro Arg Trp Phe Thr Ile Met Phe Pro Ala Met Leu Glu Leu Ala Gly
145                 150                 155                 160
Ala Ser Ser Leu Arg Val Asp Phe Ser Glu Asn Leu Asn Arg Ile Leu
                165                 170                 175
Val Glu Leu Ser Gln Asn Arg Asp Asp Ile Leu Thr Arg Glu Glu Val
            180                 185                 190
Asp Glu Lys Lys Gln Tyr Ser Pro Leu Leu Leu Phe Leu Glu Ala Leu
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        195                 200                 205
Pro Ala Gln Ser Tyr Asp Asn Asp Val Leu Lys Gln Ile Ile Asp Lys
    210                 215                 220
Asn Leu Ser Asn Asp Gly Ser Leu Leu Gln Ser Pro Ser Ala Thr Ala
225                 230                 235                 240
Arg Ala Tyr Met Ile Thr Gly Asn Thr Arg Cys Leu Ser Tyr Leu His
                245                 250                 255
Ser Leu Thr Asn Ser Cys Ser Asn Gly Gly Val Pro Ser Phe Tyr Pro
            260                 265                 270
Val Asp Asp Asp Leu His Asp Leu Val Met Val Asn Gln Leu Thr Arg
        275                 280                 285
Ser Gly Leu Thr Glu His Leu Ile Pro Glu Ile Asp His Leu Leu Leu
    290                 295                 300
Lys Val Gln Lys Asn Tyr Lys Tyr Lys Lys Ala Ser Pro Lys Ser Leu
305                 310                 315                 320
Tyr Ser Ile Ala Ala Glu Leu Tyr Arg Asp Ser Leu Ala Phe Trp Leu
                325                 330                 335
Leu Arg Val Asn Asn His Trp Val Ser Pro Ser Ile Phe Cys Trp Phe
            340                 345                 350
Leu Asp Asp Asp Glu Ile Arg Asp His Ile Glu Thr Asn Tyr Glu Glu
        355                 360                 365
Phe Ala Ala Val Leu Leu Asn Val Tyr Arg Ala Thr Asp Leu Met Phe
    370                 375                 380
Ser Gly Glu Val Gln Leu Val Glu Ala Arg Ser Phe Ala Thr Lys Asn
385                 390                 395                 400
Leu Glu Lys Ile Leu Ala Thr Gly Asn Ile His Lys Thr Asn Ala Asp
                405                 410                 415
Ile Ser Ser Ser Leu His Lys Met Ile Glu His Glu Leu Arg Val Pro
            420                 425                 430
Trp Thr Ala Arg Met Asp His Val Glu Asn Arg Ile Trp Ile Glu Glu
        435                 440                 445
Ile Ala Ser Ser Ala Leu Trp Phe Gly Lys Ser Ser Tyr Leu Arg Leu
    450                 455                 460
Ser Cys Phe His Lys Met Ser Leu Gln Gln Leu Ala Val Lys Asn Tyr
465                 470                 475                 480
Thr Leu Arg Gln Leu Val Tyr Arg Asp Glu Leu Ala Glu Val Glu Arg
                485                 490                 495
Trp Ser Lys Glu Arg Gly Leu Cys Asp Met Gly Phe Cys Arg Glu Lys
            500                 505                 510
Thr Gly Tyr Cys Tyr Tyr Ala Phe Ala Ala Ser Thr Cys Leu Pro Trp
        515                 520                 525
Ser Ser Asp Val Arg Leu Val Leu Thr Lys Ala Ala Val Val Ile Thr
    530                 535                 540
Val Ala Asp Asp Phe Phe Asp Val Glu Gly Ser Met Val Asp Leu Glu
545                 550                 555                 560
Lys Leu Thr Asp Ala Val Arg Arg Trp Asp Ala Glu Gly Leu Gly Ser
                565                 570                 575
His Ser Lys Thr Ile Phe Glu Ala Leu Asp Asp Leu Val Asn Glu Val
            580                 585                 590
Arg Leu Lys Cys Phe Gln Gln Asn Gly Gln Asp Ile Lys Asn Asn Leu
        595                 600                 605
Gln Gln Leu Trp Tyr Glu Thr Phe His Ser Trp Leu Met Glu Ala Lys
    610                 615                 620
Trp Gly Lys Gly Leu Thr Ser Lys Pro Ser Val Asp Val Tyr Leu Gly
625                 630                 635                 640
Asn Ala Met Thr Ser Ile Ala Ala His Thr Met Val Leu Thr Ala Ser
                645                 650                 655
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Cys Leu Leu Gly Pro Gly Phe Pro Val His Gln Leu Trp Ser Gln Arg
            660                 665                 670
Arg His Gln Asp Ile Thr Ser Leu Leu Met Val Leu Thr Arg Leu Leu
        675                 680                 685
Asn Asp Ile Gln Ser Tyr Leu Lys Glu Glu Asp Glu Gly Lys Ile Asn
    690                 695                 700
Tyr Val Trp Met Tyr Met Ile Glu Asn Asn Gln Ala Ser Ile Asp Asp
705                 710                 715                 720
Ser Val Arg His Val Gln Thr Ile Ile Asn Val Lys Lys Gln Glu Phe
                725                 730                 735
Ile Gln Arg Val Leu Ser Asp Gln His Cys Asn Leu Pro Lys Ser Phe
            740                 745                 750
Lys Gln Leu His Phe Ser Cys Leu Lys Val Phe Asn Met Phe Phe Asn
        755                 760                 765
Ser Ser Asn Ile Phe Asp Thr Asp Thr Asp Leu Leu Leu Asp Ile His
    770                 775                 780
Glu Ala Phe Val Ser Pro Pro Gln Val Pro Lys Phe Lys Pro His Ile
785                 790                 795                 800
Lys Pro Pro His Gln Leu Pro Ala Thr Leu Gln Pro Pro His Gln Pro
                805                 810                 815
Gln Gln Ile Met Val Asn Lys Lys Lys Val Glu Met Val Tyr Lys Ser
            820                 825                 830
Tyr His His Pro Phe Lys Val Phe Thr Leu Gln Lys Lys Gln Ser Ser
        835                 840                 845
Gly His Gly Thr Met Asn Pro Arg Ala Ser Ile Leu Ala Gly Pro Asn
    850                 855                 860
Ile Lys Leu Cys Phe Ser
865                 870
```

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/23180

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N9/88 C12N9/10 C12N9/00 C12N15/11
A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 37102 A (PIONEER HI BRED INT) 28 November 1996 (1996-11-28) the whole document ---	1-11, 13-16, 20-30
X	WO 94 22304 A (PIONEER HI BRED INT) 13 October 1994 (1994-10-13) the whole document ---	1-7, 20-30
X	WO 95 11913 A (UNIV WASHINGTON) 4 May 1995 (1995-05-04) the whole document --- -/--	1-7, 20-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 April 2000	25. 07. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040. Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internatir l Application No
PCT/US 99/23180

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COLBY S M ET AL: "4S-LIMONENE SYNTHASE FROM THE OIL GLANDS OF SPEARMINT (MENTHA SPICATA)" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 268, no. 31, 5 November 1993 (1993-11-05), pages 23016-23024, XP002914702 ISSN: 0021-9258 the whole document	

1

INTERNATIONAL SEARCH REPORT

Inte onal application No.
PCT/US 99/23180

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

SEE ADDITIONAL SHEET

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-11, 13-18, 20-30 - partially

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: {1-11, 13-18, 20-30 partially}

A method for manipulating a metabolic pathway in a plant cell comprising transforming a plant cell with a limonene synthase-encoding nucleotide sequence set forth in SEQ ID NO: 1 and coding for a protein set forth in SEQ ID NO: 2. A method for creating or enhancing resistance to insects in a plant, a method for producing limonene synthase in a plant cell, a transformed plant cell and plant having a manipulated metabolic pathway comprising said limonene synthase.

2. Claims: {1-30 partially}

same as invention 1 but comprising a GPP synthase as set forth in SEQ ID NO: 3 and 4.

3. Claims: {1-9, 11, 13-15, 17, 18, 20-30 partially}

same as in invention but comprising a carveol synthase as set forth in SEQ ID NO: 5 and 6.

4. Claims: {1-9, 12-15, 19-30 partially}

same as invention 1 but comprising an S-linalool synthase as set forth in SEQ ID NO: 7 and 8.

INTERNATIONAL SEARCH REPORT

Info. .tion on patent family members

Internatic Application No
PCT/US 99/23180

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9637102 A	28-11-1996	AT 185043 T	15-10-1999
		AU 5879496 A	11-12-1996
		BR 9609204 A	11-05-1999
		CA 2222023 A	28-11-1996
		DE 69604494 D	04-11-1999
		DE 69604494 T	05-01-2000
		EP 0836381 A	22-04-1998
		ES 2139361 T	01-02-2000
WO 9422304 A	13-10-1994	AT 185042 T	15-10-1999
		AU 6449794 A	24-10-1994
		CA 2159735 A	13-10-1994
		DE 69420957 D	04-11-1999
		DE 69420957 T	13-01-2000
		EP 0691812 A	17-01-1996
WO 9511913 A	04-05-1995	AU 8127994 A	22-05-1995
		CA 2118071 A	29-04-1995
		EP 0730603 A	11-09-1996
		US 5871988 A	16-02-1999